

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
2 August 2001 (02.08.2001)

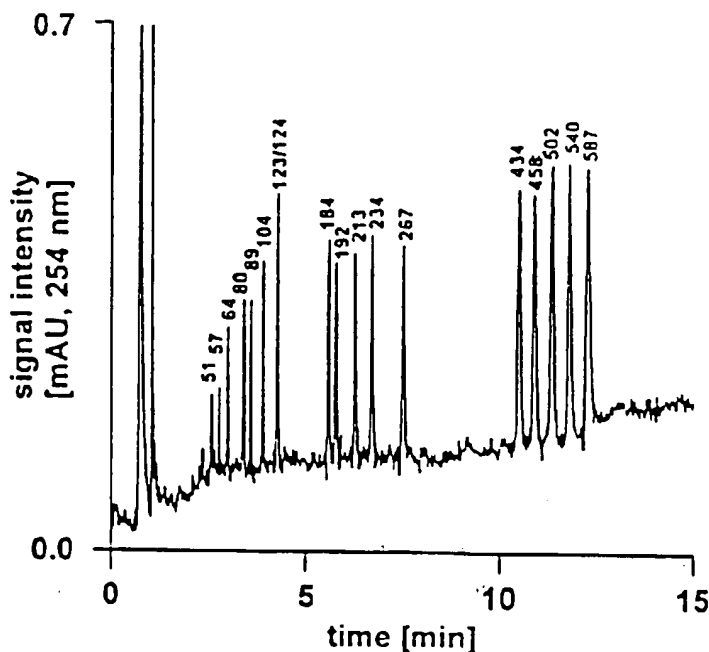
PCT

(10) International Publication Number  
WO 01/55713 A2

- (51) International Patent Classification<sup>7</sup>: G01N 30/96 (74) Agents: WALKER, William, B. et al.; Transgenomic, Inc., 2032 Concourse Drive, San Jose, CA 95131 (US).
- (21) International Application Number: PCT/US01/02539
- (22) International Filing Date: 25 January 2001 (25.01.2001) (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/178,553 26 January 2000 (26.01.2000) US
- (71) Applicant: TRANSGENOMIC, INC. [US/US]; 2032 Concourse Drive, San Jose, CA 95131 (US) (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
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[Continued on next page]

(54) Title: METHOD AND APPARATUS FOR SEPARATING POLYNUCLEOTIDES USING MONOLITHIC CAPILLARY COLUMNS



(57) Abstract: Methods and devices based on capillary monolithic columns, preferably consisting of an underivatized poly(styrene-divinylbenzene) monolith, for separating a mixture of polynucleotides by ion pair-reverse phase-high performance chromatography (IP-RP-HPLC). In various aspects of the method and device the monolith is characterized by one or more of the following: the monolith is contained within a capillary tube; the monolith is immobilized by covalent attachment at the inner wall of the tube; the tube is devoid of retaining frits; the monolith is characterized by having above 10,000 theoretical plates per meter and preferably above 200,000 theoretical plates per meter; the method uses a mobile phase which is devoid of EDTA; the monolith has a surface morphology that is rugulose or brush-like; the chromatographic surfaces of the monolith are non-porous; the monolith has channels sufficiently large for convective flow of the mobile phase;

the monolith is formed from a polymerization mixture including underivatized styrene, a crosslinking agent, and a porogen, wherein the porogen includes tetrahydrofuran. The monolith can be incorporated into a miniaturized chromatography system which can be coupled to a mass spectrometer for on-line separation and mass determination of single- or double-stranded polynucleotides.



**Published:**

— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**TITLE OF THE INVENTION**

METHOD AND APPARATUS FOR SEPARATING POLYNUCLEOTIDES USING  
MONOLITHIC CAPILLARY COLUMNS

**FIELD OF THE INVENTION**

The present invention relates to methods and devices for analyzing polynucleotides. In particular, the invention relates to the use of monolithic capillary columns for use in high-performance liquid chromatography of single and double-stranded polynucleotides.

**BACKGROUND OF THE INVENTION**

Genetics and proteomics depend on the ability to analyze complex mixtures of biological origin with high sensitivity and maximum selectivity. Especially the rapid development of miniaturized techniques in analytical chemistry (He et al. *Anal. Chem.* 70:3790-3797 (1998)) has had a profound impact on the modern practice of analyzing biological samples of high complexity (Novotny *J. Chromatogr. B* 689: 55-70 (1997)). Several techniques based on the principle of differential migration (Rathore et al. *J. Chromatogr. A* 743: 231-246 (1996)) were developed after the introduction of fused silica capillaries to analytical chemistry (Dandeneau et al. *HRC & CC*: 2:351 (1979)), in particular capillary liquid chromatography (CLC) (Hirata et al. *J. Chromatogr.* 186:521-528 (1979)), capillary electrophoresis (CE) (Jorgenson et al. *J. Chromatogr.* 218:209-216 (1981)), and capillary electrochromatography (CEC) (Jorgenson et al. *J. Chromatogr.* 218:209-216 (1981)).

Columns packed with microparticulate sorbents have been successfully applied as separation media in high-performance liquid chromatography (HPLC). Despite many advantages, HPLC columns packed with microparticulate, porous stationary phases have some limitations, such as the relatively large void volume between the packed particles and the slow diffusional mass transfer of solutes into and out of the stagnant mobile phase present in the pores of the separation medium (Martin et al. *Biochem J.* 35:1358 (1941); Unger et al in *Packings and Stationary Phases in Chromatographic Techniques*, Unger Ed: Marcel Dekker: New York, p. 75 (1990)).

One approach to alleviate the problem of restricted mass transfer and intraparticle void volume is the concept of monolithic chromatographic beds, where the separation medium consists of a continuous rod of a rigid, polymer which has no interstitial volume but only internal porosity consisting of micropores and macropores. Monolithic separation columns are becoming more widely used in HPLC of biomolecules.

WO 97/19347 relates to a method and device for separating one or several organic substances in a sample. The chromatographic device comprises a monolith prepared in an emulsion system containing at least 75% by weight of water phase. Separations of polynucleotides were not disclosed.

U.S. 5,334,310 relates to a monolith containing small pores having diameters less than about 200 nm and large pores with diameters greater than about 600 nm. The columns were equipped with end fittings. No separations of polynucleotides were demonstrated.

WO 00/15778 relates to polymeric monolithic beds for resolving mixtures containing polynucleotides. However, single-stranded molecules were poorly resolved using the column. The columns had inner diameters (ID) of greater than 4mm and were equipped retaining frits. The mobile phase buffers included EDTA. Useful separations of DNA fragments by IP-RP-HPLC using underivatized polystyrene/divinylbenzene monolithic columns could not be achieved and such columns were not recommended.

There is a need for improved monolithic columns and methods for the separation of polynucleotides.

## SUMMARY OF THE INVENTION

In one aspect, the present invention provides a method for separating a mixture of polynucleotides. The method includes applying the mixture of polynucleotides to a polymeric monolith having non-polar chromatographic surfaces and eluting the mixture of polynucleotides with a mobile phase including a counterion agent and an organic solvent, wherein the monolith is an underivatized poly(styrene-divinylbenzene) matrix. In the method, the monolith preferably is contained within a fused silica tube having an inner diameter in the range of 1 to 1000 micrometer and the monolith is immobilized by covalent attachment at the inner wall of the tube. The tube is preferably devoid of retaining frits. In preferred embodiments of this aspect of the invention, the monolith is characterized by having 100,000 to 200,000 theoretical plates per meter. The theoretical plates per meter can be determined from the retention time of single stranded p(dT)<sub>18</sub> standard using the following equation:

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half height, and  $L$  is the length of the monolith in meters. In one embodiment, during the isocratic

1 elution, the back pressure was about 180 to 200 bar, at a flow rate in the range of 2 to 3  
2  $\mu\text{L}/\text{min}$  and at an elution temperature of  $50^{\circ}\text{C}$  for a monolith having an ID of 200  
3 micrometer and a length of 60 mm. The method can be performed using a mobile  
4 phase which is devoid of EDTA. The preferred monolith has a surface morphology, as  
5 determined by scanning electron microscopy, that resembles the surface morphology of  
6 octadecyl modified poly(styrene-divinylbenzene) particles, wherein the surface  
7 morphology of the monolith is rugulose. Additionally, the preferred monolith has a  
8 surface morphology, as determined by scanning electron microscopy, that resembles  
9 the surface morphology of octadecyl modified poly(styrene-divinylbenzene) particles,  
10 wherein the surface morphology of the monolith is brush-like. The monolith can be  
11 formed from a polymerization mixture including underivatized styrene, a crosslinking  
12 agent, and a porogen, wherein the porogen includes tetrahydrofuran. A preferred  
13 porogen includes a mixture of tetrahydrofuran and decanol. In the method, the  
14 polynucleotides can include double-stranded fragments having lengths in the range of 3  
15 to 600 base pairs. The method can further include analyzing eluted polynucleotides by  
16 mass spectral analysis. In the method, the monolith preferably has a back pressure in  
17 the range of about 20 to about 300 bar, and typically in the range of about 70 to about  
18 200 bar. The method can be performed at a monolith temperature in the range of about  
19  $20^{\circ}\text{C}$  to about  $90^{\circ}\text{C}$ .

20 In another aspect, the invention concerns a method for separating a mixture of  
21 polynucleotides. The method includes applying the mixture of polynucleotides to a  
22 polymeric monolith having non-polar chromatographic surfaces and eluting the mixture  
23 of polynucleotides with a mobile phase comprising a counterion agent and an organic  
24 solvent. In a preferred embodiment, the monolith comprises an underivatized  
25 poly(styrene-divinylbenzene) matrix. In this aspect of the invention, the monolith is  
26 preferably contained within a fused silica tube, and the monolith is immobilized by  
27 covalent attachment at the inner wall of the tube. The tube can have an inner diameter  
28 in the range of 10 micrometer to 1000 micrometer, and preferably in the range of 1  
29 micrometer to 1000 micrometer. The tube is preferably devoid of retaining frits. In  
30 certain embodiments, the monolith is characterized by having 10,000 to 200,000  
31 theoretical plates per meter and preferably characterized by having 100,000 to 200,000  
32 theoretical plates per meter. During the elution, the mobile phase preferably is devoid of  
33 EDTA. The preferred monolith has a surface morphology, as determined by scanning  
34 electron microscopy, that resembles the surface morphology of octadecyl modified  
35 poly(styrene-divinylbenzene) particles, wherein the surface morphology of the monolith

1 is rugulose. The monolith can be formed from a polymerization mixture including  
2 underivatized styrene, a crosslinking agent, and a porogen, wherein the porogen  
3 comprises tetrahydrofuran.

4 In another aspect, the invention provides a method for separating a mixture of  
5 polynucleotides. The method includes applying the mixture of polynucleotides to a  
6 polymeric monolith having non-polar chromatographic surfaces and eluting the mixture  
7 of polynucleotides with a mobile phase comprising a counterion agent and an organic  
8 solvent, wherein the monolith comprises an underivatized poly(styrene-divinylbenzene)  
9 matrix, wherein the monolith is contained within a fused silica tube, and wherein the  
10 tube is devoid of retaining frits, wherein the tube has an inner diameter in the range of 1  
11 micrometer to 1000 micrometer, and wherein the polynucleotides are double-stranded  
12 fragments having lengths in the range of 3 to 600 base pairs. During the elution, the  
13 mobile phase preferably is devoid of EDTA. The monolith preferably is immobilized by  
14 covalent attachment at the inner wall of the tube. In certain embodiments, the monolith  
15 is characterized by having 50,000 to 200,000 theoretical plates per meter. In preferred  
16 embodiments, the monolith is characterized by having greater than about 190,000  
17 theoretical plates per meter. The preferred monolith has a surface morphology, as  
18 determined by scanning electron microscopy, that resembles the surface morphology of  
19 octadecyl modified poly(styrene-divinylbenzene) particles, wherein the surface  
20 morphology of the monolith is rugulose.

21 In a further aspect, the invention provides a method for separating a mixture of  
22 polynucleotides. The method includes applying the mixture of polynucleotides to a  
23 polymeric monolith having non-polar chromatographic surfaces and eluting said mixture  
24 of polynucleotides with a mobile phase comprising a counterion agent and an organic  
25 solvent, wherein the monolith is characterized by having 10,000 to 200,000 theoretical  
26 plates per meter, wherein the monolith includes an underivatized poly(styrene-  
27 divinylbenzene) matrix, wherein the monolith is contained within a fused silica tube  
28 having an inner diameter in the range of 1 micrometer to 1000 micrometer, and wherein  
29 the monolith is immobilized by covalent attachment at the inner wall of the tube. In a  
30 preferred embodiment, the theoretical plates per meter is determined from the retention  
31 time of single stranded p(dT)<sub>18</sub> standard using the following equation:

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

1 wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
2 standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half height, and  
3  $L$  is the length of the monolith in meters. The tube preferably is devoid of retaining frits.  
4 In the method, the mobile phase preferably is devoid of EDTA. In a preferred  
5 embodiment, the monolith has a surface morphology, as determined by scanning  
6 electron microscopy, that resembles the surface morphology of octadecyl modified  
7 poly(styrene-divinylbenzene) particles, wherein the surface morphology of said monolith  
8 is rugulose. Also in a preferred embodiment, the monolith has a surface morphology, as  
9 determined by scanning electron microscopy, that resembles the surface morphology of  
10 octadecyl modified poly(styrene-divinylbenzene) particles, wherein the surface  
11 morphology of said monolith is brush-like.

12 In a yet further aspect, the invention concerns a method for separating a mixture  
13 of polynucleotides. The method includes applying the mixture of polynucleotides to a  
14 polymeric monolith having non-polar chromatographic surfaces and eluting the mixture  
15 of polynucleotides with a mobile phase comprising a counterion agent and an organic  
16 solvent, and wherein the mobile phase is devoid of EDTA. In this aspect, the monolith  
17 preferably is contained within a fused silica tube having an inner diameter in the range  
18 of 10 micrometer to 1000 micrometer. The monolith preferably is immobilized by  
19 covalent attachment at the inner wall of the tube. The tube preferably is devoid of  
20 retaining frits. In certain embodiments of this aspect of the invention, the monolith is  
21 characterized by having 10,000 to 200,000 theoretical plates per meter. The preferred  
22 monolith has a surface morphology, as determined by scanning electron microscopy,  
23 that resembles the surface morphology of octadecyl modified poly(styrene-  
24 divinylbenzene) particles, wherein the surface morphology of the monolith is rugulose.  
25 The preferred monolith comprises an underivatized poly(styrene-divinylbenzene) matrix.

26 In a still further aspect, the invention provides a method for separating a mixture  
27 of polynucleotides. The method includes applying the mixture of polynucleotides to a  
28 polymeric monolith having non-polar chromatographic surfaces and eluting the mixture  
29 of polynucleotides with a mobile phase comprising a counterion agent and an organic  
30 solvent, wherein the monolith has a surface morphology, as determined by scanning  
31 electron microscopy, that resembles the surface morphology of octadecyl modified  
32 poly(styrene-divinylbenzene) particles, wherein the surface morphology of the monolith  
33 is rugulose, and wherein the monolith comprises an underivatized poly(styrene-  
34 divinylbenzene) matrix. The mobile phase preferably is devoid of EDTA. In preferred  
35 embodiments, the monolith can be characterized by one or more of the following: the

1 monolith is contained within a fused silica tube having an inner diameter in the range of  
2 1 micrometer to 1000 micrometer; the monolith is immobilized by covalent attachment at  
3 the inner wall of the tube; and, the tube is devoid of retaining frits. In preferred  
4 embodiments, the monolith is characterized by having 100,000 to 200,000 theoretical  
5 plates per meter.

6 In a related aspect, the invention provides a method for separating a mixture of  
7 polynucleotides. In this aspect, the method includes applying the mixture of  
8 polynucleotides to a polymeric monolith having non-polar chromatographic surfaces and  
9 eluting the mixture of polynucleotides with a mobile phase comprising a counterion  
10 agent and an organic solvent, wherein the monolith comprises an underivatized  
11 poly(styrene-divinylbenzene) matrix, wherein the monolith is contained within a fused  
12 silica tube having an inner diameter in the range of 1 micrometer to 1000 micrometer,  
13 wherein the monolith is immobilized at the inner wall of the tube, and wherein the tube is  
14 devoid of retaining frits. Preferred embodiments of this aspect of the invention can  
15 include one or more of the following: the mobile phase is devoid of EDTA; the monolith  
16 is characterized by having 100,000 to 200,000 theoretical plates per meter; and, the  
17 monolith has a surface morphology, as determined by scanning electron microscopy,  
18 that resembles the surface morphology of octadecyl modified poly(styrene-  
19 divinylbenzene) particles, wherein the surface morphology of the monolith is rugulose.  
20 The monolith can be formed from a polymerization mixture including underivatized  
21 styrene, a crosslinking agent, and a porogen, wherein the porogen comprises  
22 tetrahydrofuran. The method can further include analyzing eluted polynucleotides by  
23 mass spectral analysis.

24 In an additional aspect, the invention provides a device for separating a mixture  
25 of polynucleotides. The device includes a polymeric monolith having non-polar  
26 chromatographic surfaces, wherein the monolith is contained within a fused silica tube  
27 having an inner diameter in the range of 1 micrometer to 1000 micrometer, wherein the  
28 monolith is immobilized by covalent attachment at the inner wall of the tube, and  
29 wherein the monolith comprises an underivatized poly(styrene-divinylbenzene) matrix.  
30 Preferred embodiments of this aspect of the invention can be further characterized by  
31 the following: the tube is devoid of retaining frits; the monolith is characterized by having  
32 100,000 to 200,000 theoretical plates per meter. The theoretical plates per meter  
33 preferably is determined from the retention time of single stranded p(dT)<sub>18</sub> standard  
34 using the following equation:

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

1           wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
2 standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half height, and  
3  $L$  is the length of the monolith in meters. During the isocratic elution the monolith  
4 preferably has a back pressure of 180 to 200 bar, and a flow rate in the range of 2 to 3  
5  $\mu\text{L}/\text{min}$  at an elution temperature of  $50^\circ\text{C}$ . Preferred embodiments of the device can be  
6 characterized by one or more of the following: the chromatographic surfaces of the  
7 monolith are non-porous; the monolith has channels sufficiently large for convective flow  
8 of said mobile phase; and, the monolith can be formed from a polymerization mixture  
9 including underivatized styrene, a crosslinking agent, and a porogen, wherein the  
10 porogen comprises tetrahydrofuran.

11           In a further yet aspect, the invention concerns a device for separating a mixture  
12 of polynucleotides. The device includes a polymeric monolith having non-polar  
13 chromatographic surfaces, wherein the monolith is contained within a fused silica tube,  
14 wherein the monolith is immobilized by covalent attachment at the inner wall of the tube,  
15 and wherein the monolith comprises an underivatized poly(styrene-divinylbenzene)  
16 matrix. Preferred embodiments can include one or more of the following features: the  
17 tube has an inner diameter in the range of 1 micrometer to 1000 micrometer; the tube is  
18 devoid of retaining frits; the monolith is characterized by having 10,000 to 200,000  
19 theoretical plates per meter; the monolith comprises an underivatized monolithic  
20 stationary phase; the monolith has a surface morphology, as determined by scanning  
21 electron microscopy, that resembles the surface morphology of octadecyl modified  
22 poly(styrene-divinylbenzene) particles, wherein the surface morphology of the monolith  
23 is rugulose; the chromatographic surfaces of the monolith are non-porous; and the  
24 particles have channels sufficiently large for convective flow of the mobile phase. The  
25 monolith can be formed from a polymerization mixture including underivatized styrene, a  
26 crosslinking agent, and a porogen, wherein the porogen comprises tetrahydrofuran.

27           In another aspect, the invention concerns a device for separating a mixture of  
28 polynucleotides. The device includes a polymeric monolith having non-polar  
29 chromatographic surfaces, wherein the monolith is contained within a fused silica tube,  
30 wherein the tube is devoid of retaining frits, and wherein the monolith comprises an  
31 underivatized poly(styrene-divinylbenzene) matrix. Preferred embodiments of this  
32 aspect of the invention can further include one or more of the following: the monolith is

1 immobilized by covalent attachment at the inner wall of said tube; the monolith is  
2 characterized by having 100,000 to 200,000 theoretical plates per meter; the tube has  
3 an inner diameter in the range of 1 micrometer to 1000 micrometer; and, the monolith  
4 has a surface morphology, as determined by scanning electron microscopy, that  
5 resembles the surface morphology of octadecyl modified poly(styrene-divinylbenzene)  
6 particles, wherein the surface morphology of the monolith is rugulose. The monolith can  
7 be formed from a polymerization mixture including underivatized styrene, a crosslinking  
8 agent, and a porogen, wherein the porogen comprises tetrahydrofuran.

9 In a related aspect, the invention provides a device for separating a mixture of  
10 polynucleotides. The device includes a polymeric monolith having non-polar  
11 chromatographic surfaces, wherein the monolith is characterized by having 100,000 to  
12 200,000 theoretical plates per meter, wherein the monolith is contained within a fused  
13 silica tube having an inner diameter in the range of 1 micrometer to 1000 micrometer,  
14 and wherein the tube has been silianized. Preferred embodiments of this aspect of the  
15 invention can further include one or more of the following: the monolith is immobilized  
16 by covalent attachment at the inner wall of the tube; the tube is devoid of retaining frits;  
17 the monolith comprises an underivatized poly(styrene-divinylbenzene) matrix; and, the  
18 monolith has a surface morphology, as determined by scanning electron microscopy,  
19 that resembles the surface morphology of octadecyl modified poly(styrene-  
20 divinylbenzene) particles, wherein the surface morphology of the monolith is rugulose.  
21 The monolith can be formed from a polymerization mixture including underivatized  
22 styrene, a crosslinking agent, and a porogen, wherein the porogen comprises  
23 tetrahydrofuran.

24 In an important aspect, the invention provides a device for separating a mixture  
25 of polynucleotides. The device includes a polymeric monolith having non-polar  
26 chromatographic surfaces, wherein the monolith comprises an underivatized  
27 poly(styrene-divinylbenzene) matrix, and wherein the monolith is characterized by  
28 having 10,000 to 200,000 theoretical plates per meter. Preferred embodiments of this  
29 aspect of the invention can further include one or more of the following: the monolith is  
30 contained within a tube having an inner diameter in the range of 1 micrometer to 1000  
31 micrometer; the monolith is immobilized at the inner wall of the tube; the tube is devoid  
32 of retaining frits; and, the monolith has a surface morphology, as determined by  
33 scanning electron microscopy, that resembles the surface morphology of octadecyl  
34 modified poly(styrene-divinylbenzene) particles, wherein the surface morphology of the  
35 monolith is rugulose.

1 In another aspect, the invention provides a miniaturized chromatographic system  
2 for separating a mixture of polynucleotides. The device includes a polymeric monolith  
3 having non-polar chromatographic surfaces, wherein the monolith comprises an  
4 underivatized poly(styrene-divinylbenzene) matrix, wherein the monolith is  
5 characterized by having at least 100,000 theoretical plates per meter, wherein the  
6 monolith is contained within a tube having an inner diameter in the range of 10  
7 micrometer to 1000 micrometer, and wherein the monolith is immobilized at the inner  
8 wall of the tube. Preferred embodiments of this aspect of the invention can further  
9 include one or more of the following: the tube is devoid of retaining frits; the monolith is  
10 contained within a tube having an inner diameter in the range of 1 micrometer to 1000  
11 micrometer; the monolith has a surface morphology, as determined by scanning  
12 electron microscopy, that resembles the surface morphology of octadecyl modified  
13 poly(styrene-divinylbenzene) particles, wherein the surface morphology of the monolith  
14 is rugulose; and wherein the monolith has a surface morphology, as determined by  
15 scanning electron microscopy, that resembles the surface morphology of octadecyl  
16 modified poly(styrene-divinylbenzene) particles, wherein the surface morphology of the  
17 monolith is brush-like. The monolith can be formed from a polymerization mixture  
18 including underivatized styrene, a crosslinking agent, and a porogen, wherein the  
19 porogen comprises tetrahydrofuran.

20 In an additional aspect, the invention concerns a miniaturized chromatographic  
21 system for separating a mixture of polynucleotides. The system preferably includes a  
22 device which includes a polymeric monolith having non-polar chromatographic surfaces,  
23 wherein the monolith comprises an underivatized poly(styrene-divinylbenzene) matrix,  
24 wherein the monolith is characterized by having at least 100,000 theoretical plates per  
25 meter, wherein the monolith is contained within a tube having an inner diameter in the  
26 range of 10 micrometer to 1000 micrometer, and wherein the monolith is immobilized at  
27 the inner wall of the tube. In the system, the monolith can be operatively coupled to a  
28 mass spectrometer.

29  
30 In a further aspect, the invention concerns a device for separating a mixture of  
31 polynucleotides. The device includes a polymeric monolith having non-polar  
32 chromatographic surfaces, wherein the monolith has a surface morphology, as  
33 determined by scanning electron microscopy, that resembles the surface morphology of  
34 octadecyl modified poly(styrene-divinylbenzene) particles, wherein the surface  
35 morphology of the monolith is rugulose and brush-like, wherein the monolith is

1 contained within a fused silica tube having an inner diameter in the range of 1  
2 micrometer to 1000 micrometer, and wherein the monolith is immobilized at the inner  
3 wall of said tube. Preferred embodiments of this aspect of the invention can further  
4 include one or more of the following: the tube is devoid of retaining frits; the monolith is  
5 characterized by having 100,000 to 200,000 theoretical plates per meter; the monolith  
6 comprises an underivatized poly(styrene-divinylbenzene) matrix; and, the surface of  
7 said monolith is non-porous. The monolith can be formed from a polymerization mixture  
8 including underivatized styrene, a crosslinking agent, and a porogen, wherein the  
9 porogen comprises tetrahydrofuran. The polynucleotides can include double-stranded  
10 fragments having lengths in the range of 3 to 2000 base pairs, and preferably 3 to 600  
11 base pairs.

12 In a final aspect, the invention concerns a chromatographic device. The device  
13 includes a polymeric monolith having non-polar chromatographic surfaces, wherein the  
14 monolith comprises an underivatized poly(styrene-divinylbenzene) matrix, wherein the  
15 monolith is characterized by having at least 10,000 theoretical plates per meter, wherein  
16 the monolith is contained within a silanized fused silica tube having an inner diameter in  
17 the range of 10 micrometer to 1000 micrometer, and wherein the monolith is  
18 immobilized at the inner wall of the tube. Preferred embodiments of this aspect of the  
19 invention can be further characterized by the following: the tube is devoid of retaining  
20 frits; the monolith is characterized by having 100,000 to 200,000 theoretical plates per  
21 meter; the monolith has a surface morphology, as determined by scanning electron  
22 microscopy, that resembles the surface morphology of octadecyl modified poly(styrene-  
23 divinylbenzene) particles, wherein the surface morphology of the monolith is rugulose;  
24 and wherein the monolith has a surface morphology, as determined by scanning  
25 electron microscopy, that resembles the surface morphology of octadecyl modified  
26 poly(styrene-divinylbenzene) particles, wherein the surface morphology of the monolith  
27 is brush-like. The monolith can be formed from a polymerization mixture including  
28 underivatized styrene, a crosslinking agent, and a porogen, wherein the porogen  
29 comprises tetrahydrofuran. The theoretical plates per meter preferably is determined  
30 from the retention time of single stranded p(dT)<sub>18</sub> standard using the following equation:

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

31 wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
32 standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half height, and

1  $L$  is the length of the monolith in meters. During the isocratic elution the monolith  
2 preferably has a back pressure of 180 to 200 bar, and a flow rate in the range of 2 to 3  
3  $\mu\text{L}/\text{min}$  at an elution temperature of  $50^{\circ}\text{C}$ . Preferred embodiments of the device can be  
4 characterized by one or more of the following: the chromatographic surfaces of the  
5 monolith are non-porous; the monolith has channels sufficiently large for convective flow  
6 of said mobile phase; and, the monolith can be formed from a polymerization mixture  
7 including underivatized styrene, a crosslinking agent, and a porogen, wherein the  
8 porogen comprises tetrahydrofuran. The device can be used with back pressures in the  
9 range of about 20 to 300 bar, and with temperatures in the range of  $20^{\circ}\text{C}$  to about  $90^{\circ}\text{C}$ .

#### 10 BRIEF DESCRIPTION OF THE DRAWINGS

11 FIG. 1 illustrates a method for derivatization of a capillary silica wall by (a) vinyl-  
12 silanization and (b) subsequent grafting of the forming polymer.

13 FIG. 2 is a chromatogram showing capillary ion-pair reverse phase-high  
14 pressured liquid chromatography (IP-RP-HPLC) separation of phosphorylated  
15 polynucleotide ladders (0.66 - 1.64 fmol of each polynucleotide) in a monolithic capillary  
16 column constructed in accordance with an embodiment of the present invention.

17 FIG. 3 is a chromatogram showing capillary ion-pair reverse phase-high  
18 pressured liquid chromatography (IP-RP-HPLC) separation of phosphorylated  
19 polynucleotide ladders (40 - 98 fmol of each polynucleotide) in a monolithic capillary  
20 column constructed in accordance with an embodiment of the present invention.

21 FIG. 4 is a chromatogram showing capillary IP-RP-HPLC separation of  
22 phosphorylated and dephosphorylated deoxyadenylic acids in a monolithic capillary  
23 column constructed in accordance with an embodiment of the present invention.

24 FIG. 5 is a chromatogram showing capillary IP-RP-HPLC separation of a mixture  
25 of double-stranded DNA fragments in a monolithic capillary column constructed in  
26 accordance with an embodiment of the present invention. The sample was a pBR322-  
27 *Hae* III digest, 4.5 fmol of each fragment.

28 FIG. 6 is a chromatogram showing capillary IP-RP-HPLC separation of a mixture  
29 of double-stranded DNA fragments in a monolithic capillary column constructed in  
30 accordance with an embodiment of the present invention. The sample was a pBR322-  
31 *Hae* III digest, 1.81 fmol of each fragment.

32 FIG. 7 shows a scanning electron micrograph of underivatized PS-DVB particles.

33 FIG. 8 shows a scanning electron micrograph of octadecylated PS-DVB particles.

34 FIG. 9 shows a scanning electron micrograph of an underivatized PS-DVB  
35 monolith.

FIG. 10 illustrates the separation and mass analysis of a series of oligothymidylic acids.

FIG. 11 is a chromatogram showing analysis of a crude synthetic 80-mer oligodeoxynucleotide by on-line IP-RP-HPLC-ESI-MS.

FIG. 12 is a chromatogram showing extraction of selected ion chromatograms from the data shown in FIG. 11.

FIG. 13 is a chromatogram showing averaging and deconvolution of four mass spectra between 3.7 and 3.8 min from FIG. 11.

FIG. 14 is a chromatogram showing the separation and mass analysis of double-stranded DNA fragments from a *Hae* III digest of pBR322 plasmid (180 fmol of each fragment).

FIG. 15 shows extracted and deconvoluted mass spectra of the 80 pb fragment of the pBR322 DNA-*Hae* III digest under the same analysis conditions as in FIG. 14.

FIG. 16 shows extracted and deconvoluted mass spectra of the 123/124 pb fragment of the pBR322 DNA-*Hae* III digest under the same analysis conditions as in FIG. 14.

FIG. 17 shows extracted and deconvoluted mass spectra of the 267 pb fragment of the pBR322 DNA-*Hae* III digest under the same analysis conditions as in FIG. 14.

FIG. 18 illustrates IP-RP-HPLC-MS analysis of an unfragmented 5-mer oligodeoxynucleotide.

FIG. 19 shows a mass spectrum obtained from IP-RP-HPLC-ESI-MS/MS analysis.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention generally relates to column chromatography. The chromatographic separation is carried out by forcing a liquid through a column packed with a monolithic matrix. A sample, such as a mixture of one or more polynucleotides, is introduced at the top of the column and then moves with the flow through the column. The polynucleotides are retarded on the matrix in such a manner that polynucleotides having different lengths are retarded differently during elution using a mobile phase gradient of organic solvent.

In its most general form, the invention concerns the separation of polynucleotides. e.g. DNA, utilizing a stationary separation medium having non-polar surfaces. The separation is performed on the stationary surface. Any surface micropores preferably are of a size which excludes the smallest polynucleotide being analyzed. In the invention, the separation surfaces comprise the surfaces of interstitial

spaces in a molded polymeric monolith. The preferred separation medium is in the form of a polymeric monolith such as a monolithic rod. The monolith is polymerized or formed as a single unit inside of a tube. The channels (i.e., through-pores or macropores) provide for the passage of eluting solvent and analyte materials. The separation is performed on the stationary surface. All of the mobile phase is forced to flow through the channels of the separation medium (Petro et al. *J. Chromatogr. A* 752:59-66 (1996)). Without wishing to be bound by any particular theory, it is believed that mass transport is enhanced by such convection (Rodrigues et al. *J. Chromatogr.* 653:189 (1993); Liapis, *Math. Modelling Sci. Comput.* 1:397 (1993); Liapis et al. *J. Chromatogr. A* 660:85 (1994)) and has a positive effect on chromatographic efficiency (Afeyan et al. *J. Chromatogr.* 519:1-29 (1990)).

As used herein, the term "non-porous" is defined to include a monolithic separation surface which has surface micropores having a diameter that is less than the size and shape of the smallest polynucleotide fragment in the separation in the solvent medium used therein. Included in this definition are separation surfaces having these specified maximum size restrictions in their natural state or which have been treated to reduce their micropore size to meet the maximum effective micropore size required.

The surface conformations of monoliths of the present invention can include depressions and shallow pit-like structures which do not interfere with the separation process. A pretreatment of a porous monolith to render it non-porous can be effected with any material which will fill the micropores in the surface of the monolith structure and which does not significantly interfere with the IP-RP-HPLC process. "IP-RP-HPLC" includes a process for separating single and double-stranded polynucleotides using a non-polar separation medium, wherein the process uses a counterion agent, and an organic solvent to elute the nucleic acid from the non-polar surface of the medium.

As used herein, the term "polynucleotide" includes reference to a polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), which can be single- or double-stranded, optionally incorporating synthetic, non-natural, or altered nucleotides capable of incorporation into DNA or RNA polymers, e.g., methylated nucleotides and nucleotide analogs. Polynucleotides may have any three-dimensional structure, and may optionally be partially or fully denatured. The following are non-limiting examples of polynucleotides: a gene or gene fragment (e.g., restriction fragments), exons, introns, messenger RNA, transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

1 In a general aspect, the invention provides a chromatographic system for separating  
2 a mixture of polynucleotides. The system typically includes a separation column, a  
3 source of mobile phase, a pump, an injector, a column oven, a detector, a fraction  
4 collector, and a computer system including control software.

5 In a preferred embodiment of the instant invention, the chromatographic system  
6 utilizes miniaturized system components and column tubing having small inner  
7 diameters (e.g., having an ID of 1 micrometer to 5,000 micrometer, typically having and  
8 ID of 1 micrometer to 1,000 micrometer, and preferably having a column ID of about 10  
9 micrometer to about 500 micrometer). Four major advantages connected with the use of  
10 smaller dimensions in chromatographic separation techniques can be specified:  
11 increased mass sensitivity with concentration-sensitive detectors allows the analysis of  
12 smaller samples (Novotny); on-line conjugation to mass spectrometry is feasible  
13 (Yergey et al. *Liquid Chromatography/Mass Spectrometry-Techniques and Applications*,  
14 Plenum Press, New York (1990); Niessen et al. *Liquid Chromatography-Mass  
15 Spectrometry: Principles and Applications*, Marcel Dekker, Inc., New York, (1992));  
16 higher separation efficiency and better resolving power can be accomplished in shorter  
17 time (Karlsson et al. *Anal. Chem.* 60:1662-1665 (1988)); Kennedy et al. *Anal. Chem.*  
18 61:1128-1135 (1989)); McCloskey in *Mass Spectrometry*, Academic Press Inc., San  
19 Diego (1990)); and expenses connected with consumption of mobile and stationary  
20 phase are cut down.

21 Without wishing to be bound by theory, high efficiency of microcolumns is attributed  
22 to decreased flow dispersion and a very homogenous packing bed structure, in which  
23 the stabilizing influence of the wall is felt by the entire packing bed (Kenndey). The  
24 volume of eluent used in microcolumn chromatography is considerably reduced, which  
25 means that the solutes of interest are dissolved in much less eluent, resulting in higher  
26 mass sensitivity and easier coupling with mass spectrometry.

27 In a preferred embodiment of the instant invention, microcolumn HPLC systems are  
28 designed and operated with the utmost attention to eliminating extracolumn band  
29 dispersion attributable to the sampling volume, detection volume, connecting tubing,  
30 and system time constant (Scott et al. *J. Chromatogr. Sci.* 20:62-66 (1982); Novotny  
31 *Anal. Chem.* 60:500A-510A (1988)). Introduction of small sample volumes and amounts  
32 into microcolumns by direct injection with microinjectors ( $\geq 20$  nL), moving injection  
33 (Borra et al. *J. Chromatogr.* 395:75-85 (1987)), split injection (McGuffin et al. *Anal.*  
34 *Chem.* 55:580-583 (1983)), heart cutting injection (McGuffin et al.), or electrokinetic  
35 injection is mandatory for preventing column overloading and minimizing peak variance.

Also in a preferred embodiment of the present invention, the micro-HPLC detector is miniaturized in order to efficiently detect a narrow peak eluting from a capillary column. The detector is capable of monitoring the column effluent from capillaries with high fidelity. An example of a suitable detector is a curved capillary flow cell with improved performance for capillary HPLC (Chervet et al. *An Improved Method of and a Capillary Flow Cell for Analysing Fluid Samples*, European patent application no. 0597552A1 (1993)). In on-column detection, a section of the capillary column can be converted to the flow cell upon removing the polyimide coating and is exposed to the light beam of a conventional UV/VIS spectrophotometric detector (Chen et al. *Anal. Meth. Instr.*, 2:122-128 (1995)). Other detection methods and ancillary techniques can be used, such as conductivity, light scattering, evaporative detection, mass spectrometry (Yergey et al. (1990); Niessen et al. (1992)), electrochemical detection (Colon et al. *Anal. Chem.* 65:476 (1993); Ewing et al. *Anal. Chem.* 66:527A (1994)), radiometric detection (Tracht et al. *Anal. Chem.* 66:2382 (1994)), and multichannel fluorescence detection (Timperman et al. *Anal. Chem.* 67:139 (1995)).

Because the gradient delay volume must be kept at a minimum, carrying out gradient elution in miniaturized HPLC is more complicated than using conventional solvent delivery systems. Some modifications of commercially available solvent delivery systems include stepwise gradients (Hirata et al. *J. Chromatogr.* 186:521-528 (1979)), split-flow operation (Van der Wal et al. *J. High Res. Chromatogr.* (1983); Chervet *Micro Flow Processor*, European patent application no. 0495255A1 (1991)), preformed gradients (Davis et al. *J. Am. Soc. Mass Spectrom.* 6:571-577 (1995)), and miniaturized diluting chambers (Takeuchi et al. *J. Chromatogr.* 253:41-47 (1982); Karlsson et al. *J. Chromatogr.* 7:411-413 (1984)). Commercially available micro-HPLC instrumentation with micro-mixing chambers is capable of performing reproducible gradients with flow rates as low as 5-10  $\mu\text{L}/\text{min}$  without solvent splitting.

High pressure pumps are used for pumping mobile phase in the systems described herein. It will be appreciated that other methods are known for driving mobile phase through separation media and can be used in carrying out the separations of polynucleotides as described in the present invention. A non-limiting example of such an alternative method includes "capillary electrochromatography" (CEC) in which an electric field is applied across capillary columns packed with microparticles and the resulting electroosmotic flow acts as a pump for chromatography. Electroosmosis is the flow of liquid, in contact with a solid surface, under the influence of a tangentially applied electric field. The technique combines the advantages of the high efficiency obtained

1 with capillary electrophoretic separations, such as capillary zone electrophoresis, and  
2 the general applicability of HPLC. CEC has the capability to drive the mobile phase  
3 through columns packed with chromatographic particles, especially small particles,  
4 when using electroosmotic flow. High efficiencies can be obtained as a result of the  
5 plug-like flow profile. In the use of CEC in the present invention, solvent gradients are  
6 used and rapid separations can be obtained using high electric fields. The following  
7 references describing CEC are each incorporated in their entirety herein: Dadoo, et al,  
8 *LC-GC* 15:630 (1997); Jorgenson, et al., *J. Chromatog.* 218:209 (1981); Pretorius, et  
9 al., *J. Chromatog.* 99:23 (1974); and the following U.S. Patent Nos. to Dadoo 5,378,334  
10 (1995), 5,342,492 (1994), and 5,310,463 (1994). Another example of a method for  
11 driving mobile phase includes centrifugal force, such as described in US 6,063,589.

12 In a particular aspect, the instant invention provides a separation column that  
13 consists of a polymeric monolith having non-polar chromatographic surfaces. The  
14 process for producing the columns generally comprises (1) adding to a rigid tube sealed  
15 at both ends a deaerated polymerizable mixture containing an inert porogen; (2)  
16 polymerizing the mixture, typically in the presence of a catalyst, to form a macroporous  
17 polymer plug; and (3) washing the plug with a solvent so as to remove the porogen  
18 present in the macroporous polymer produced. The polymerizable mixture contains a  
19 suitable monomer or monomer mixture with appropriate amounts of a suitable  
20 crosslinker.

21 Macroporous matrices are obtained when polymerization and crosslinking take  
22 place in the presence of inert porogens which lead to a phase separation during the  
23 ongoing polymerization reaction and effect the formation of permanent channels in the  
24 material (Seidl et al. *Adv. Polymer Sci.*, 5:113-213 (1967); Hjerten et al. *Nature*,  
25 356:810-811 (1992); C. Viklund et al. *Chem. Mater.* 8:744-750 (1996)). The concept of  
26 monolithic stationary phases is especially favorable for the fabrication of capillary  
27 columns.

28 Applicants have found that the exact adjustment of the polymerization conditions  
29 is crucial for the preparation of high performance monoliths of the present invention.  
30 These conditions include use of an inert component, the porogen, or a mixture of such  
31 inert components that do not participate in the polymerization and which are soluble in  
32 or at least miscible with the monomer. Careful control of the polymerization kinetics is  
33 also required to model the morphology of the formed polymer. Temperature, reaction  
34 time, concentration of radical initiator, ratio of monomer to crosslinker affect the  
35 performance of the monolith.

1       The most important parameters for the construction of special channel sizes are  
2 monomer type and reactivity, degree of crosslinking, amount and type of porogen(s),  
3 solvency of the porogen(s) for the polymer, and polymerization temperature (Seidl et al.;  
4 Svec et al. *Macromolecules* 28:7580-7582 (1995); Viklund et al. *Chem. Mater.* 9:463-  
5 471 (1997); Wang et al. *Anal. Chem.* 64:1232-1238 (1992)). To avoid undesired  
6 sedimentation, the columns can be rotated slowly in the course of the polymerization  
7 process. Column permeability and performance can be modulated over a wide range  
8 by varying the amount of porogen in the polymerization mixture. For differing  
9 compositions of the porogen, the amount of radical initiator has to be newly optimized to  
10 maintain a reasonable separation performance. Monoliths with high back pressure can  
11 be obtained using high percentages of porogen, while for columns with lower back  
12 pressure a composition with high amount of initiator and a low percentage of the  
13 porogen tetrahydrofuran is preferred. Additionally, not all the pieces that are cut from  
14 one synthesized capillary monolithic column are identical and the chromatographic  
15 performance of the pieces must be determined

16       The preferred monolithic columns were synthesized to exhibit hydrodynamic  
17 properties comparable to that of packed columns. The back pressure in a 6 cm long  
18 monolithic column (prepared as described in Example 3) for water at a flow rate of 3  
19  $\mu\text{L}/\text{min}$  is was in the range of 90 to 120 bar, which compares well to a column packed  
20 with non-porous beads of equal dimensions and comparable chromatographic efficiency  
21 that exhibited a back pressure of 150 bar. The lower back pressure in monoliths is a  
22 result of increased macroporosity. The monoliths of the invention can be used at back  
23 pressures in the range of about 20 to 300 bar. The back pressure will be dependant  
24 upon the dimensions, the length and inner diameter, of the tube. In general, a shorter  
25 tube will give a lower back pressure.

26       The method preferably is performed at an elution temperature within the range of  
27 20°C to 90°C.

28       In an important aspect, the instant invention is based on the surprising and  
29 unexpected discovery that an underivatized poly(styrene-divinylbenzene) (PS-DVB)  
30 monolith exhibited highly efficient separation performance. This was unexpected, since  
31 the disclosure in the published patent application WO 00/15778, which further cited  
32 other suggestions in the literature, disclosed that underivatized  
33 poly(polystyrene/divinylbenzene) structures are not desirable for DNA separations. It  
34 was disclosed that no useful separation using such monoliths were obtained. In the

present invention, the term "underivatized", as used in describing a monolithic matrix, is used herein to indicate that the monolithic matrix is not substituted with alkyl moieties (such as straight chain, branched or aromatic hydrocarbons) or with non-alkyl moieties (such as charged or polar groups).

In preparing the monoliths of the present invention, a preferred monomer is styrene and a preferred crosslinking agent is divinylbenzene. Examples of preferred porogens include toluene, decanol, hexane and tetrahydrofuran.

Based on preliminary experiments, a ratio of monomer to porogen mixture of 2:3 was found suitable in the preparation of the monoliths of the present invention. The chemical purity of the commercially available styrene was better than 99%. However, an assay of the utilized divinylbenzene revealed that only 65% of the used reagent were indeed isomers of divinylbenzene, capable of performing the crosslinking of polymer chains, while a percentage of about 33% was formed by different ethylvinyl benzenes that can act as a monomer for polymerization, but not crosslinker. In the description herein, the true amount or percentage of chemically pure divinylbenzene is indicated, and the amount or percentage of the non crosslinking ethylvinyl benzene was added to that of styrene. The composition of mixtures is either given in absolute masses or as percentages weight-per-weight. The density of the most used reagents is given in Table 1.

Table 1

Density of the components of the polymerization mixture

| <i>component</i> | <i>density <math>\tau_{20^{\circ}\text{C}}</math> [kg/m<sup>3</sup>]</i> |
|------------------|--------------------------------------------------------------------------|
| styrene          | 909                                                                      |
| divinylbenzene   | 914                                                                      |
| 1-decanol        | 829                                                                      |
| hexane           | 660                                                                      |
| tetrahydrofuran  | 889                                                                      |
| toluene          | 867                                                                      |

In a preferred embodiment of the instant invention, the monolith is comprised of an underivatized poly(styrene-divinylbenzene) matrix. Applicants have surprisingly discovered that the porogenic solvent tetrahydrofuran gave monolithic columns displaying unexpectedly high efficiency of separation of polynucleotides. Therefore, a

1 preferred porogenic solvent includes tetrahydrofuran. A more preferred porogen solvent  
2 comprises a mixture of tetrahydrofuran and decanol.

3 An embodiment of a polymerization mixture for the synthesis of suitable columns for  
4 the separation of biopolymers included the following: 0.5948 g non-crosslinking  
5 monomer (styrene + ethylvinyl benzene), 0.2911 g crosslinker (divinylbenzene), 1.0062  
6 g 1-decanol, 0.1759 g tetrahydrofuran, and 0.0193 g  $\alpha,\alpha'$ -azobisisobutyronitrile (ABIN).  
7 Monolithic capillary columns were produced by polymerization at 70°C for 24 hours.

8 Without wishing to be bound by theory, it is believed that the improved separation  
9 performance of the monolithic columns of the instant invention is due to the use of  
10 tetrahydrofuran as microporogen, which is more polar and of poorer solvency for the  
11 polymer than the commonly used toluene. The resulting polymer contains relatively  
12 large channels that allow rapid convective mass transport between the mobile phase  
13 and a thin out layer of the polymer. This configuration adequately imitates the  
14 configuration of micropellicular, beaded stationary phases (e.g., as disclosed in US  
15 5,585,236), which have been shown to be highly suitable for high-speed separations of  
16 biopolymers.

17 In another aspect, the instant invention provides a monolith that is contained  
18 within a tube and which is immobilized at the inner wall of the tube. In a preferred  
19 embodiment, the monolith is immobilized by covalent attachment at the inner wall of the  
20 tube. Shrinking of the monolith can be an issue during polymerization. The overall  
21 volume shrinkage during polymerization of methacrylate polymers amounts to  
22 approximately 6%, and shrinkage occurs mainly at a late point in polymerization within  
23 the formed and already crosslinked monolith (Brooks *Macromol. Chem., Macromol.*  
24 *Symp.* 35/36:121 (1990)). Therefore an extension of the channels is to be expected  
25 rather than a shrinkage of the exterior dimensions and detaching from the capillary wall.  
26 Furthermore, derivatization of the capillary inner wall with vinylsilanes facilitates wetting  
27 with polymerization mixture. reduces the formation of bubbles and can be used to \_\_\_\_\_

1 was held in its place by a PEEK frit in a stainless steel union. A longitudinal  
2 compression of 2 mm out of 80 mm, corresponding to a reduction of length of 2.5%,  
3 was observed when a pressure of 200 bar was applied. The pressure-to-flow curve  
4 starts out linearly, but begins to rise exponentially as soon as compression of the  
5 monolith and thus restriction of the channels begins at a flow rate of 6  $\mu\text{L}/\text{min}$  and a  
6 respective back pressure of 25 bar (data not shown). No such compression was  
7 observed and a linear pressure-to-flow curve over the whole range of tested flow rates  
8 and applied pressure was observed with monoliths that were chemically immobilized to  
9 the surface.

10 The monolithic columns prepared as described herein can be equipped with  
11 conventional retaining frits. However, in preferred embodiments, the monolithic  
12 columns of the invention are devoid of retaining frits. Thus in another aspect, the  
13 invention provides a polymeric monolith; preferably an underivatized poly(styrene-  
14 divinylbenzene) monolith, that is contained within a tube wherein the tube is devoid of  
15 retaining frits. In preferred embodiments, the monolith is immobilized at the capillary  
16 wall during polymerization. Such immobilization eliminates the necessity to prepare a  
17 tiny retaining frit, which is one of the more tedious and difficult to control steps during  
18 the manufacture of packed bed capillary columns (Svec et al. *Macromolecules* 28:7580-  
19 7582 (1995); C. Ericson et al. *J. Chromatogr. A* 767:33-41 (1997); Oberacher et al. *J.*  
20 *Chromatogr. A* 893:23-35 (2000)). Capillary columns prepared without frits are thus  
21 easier to prepare and less expensive.

22 After polymerization is complete, the solid monolith is preferably washed to  
23 remove any porogenic solvent and with a suitable solvent to dissolve any soluble  
24 polymer present. Suitable washing solvents include methanol, ethanol, benzene,  
25 acetonitrile, toluene acetone, tetrahydrofuran, and dioxane. This washing process may  
26 be done in stages; for example by alternatively washing with solvent and water, or by  
27 continuous washing with a solvent. The washing step is performed by pumping the  
28 solvent through the tube filled with the monolith.

29 A wide variety of conventional support structures, such as a tube, a channel or  
30 groove on a plate, a thin film across a plate, or a microchip, can be used with the  
31 monolithic matrix of the instant invention. Examples of such structures are described,  
32 for example, in WO 00/15778.

33 A still further aspect of the instant invention concerns the morphology of the  
34 surface structure of the monolith. The morphology of the synthesized monolithic  
35 polymers was optically characterized by light microscopy and by scanning electron

1 microscopy. The homogeneity of the monolithic stationary phase over the length of the  
2 capillary was controlled using an Olympus BH-2 light microscope (magnification factor  
3 from 40 to 1,000). Electron micrographs were acquired using a Voyager ARL-SEM-Q-  
4 electron micrograph (Noran Instruments Inc., Middleton, WI) with a magnification factor  
5 from 200 to 30,000.

6 Scanning electron micrographs were acquired to characterize the column  
7 morphology and surface structure. FIG. 9 shows scanning electron micrographs of the  
8 stationary phase made of a highly crosslinked, underivatized, styrene-divinylbenzene  
9 copolymer monolith. The cross section of the rod reveals clusters of globules separated  
10 by large channels. The average size of the globules is in the range of 100 to 200 nm,  
11 they form the building units for larger aggregates with a diameter from 500 to 800 nm.  
12 The size of the channels between the clusters reaches 500 nm, corresponding well to  
13 those measured by inverse size exclusion chromatography.

14 The surface structure of the poly(styrene-divinylbenzene) monoliths of the present  
15 invention was compared to that of octadecyl modified poly(styrene-divinylbenzene)  
16 particles, which have been shown to be highly suitable for high-speed separation of  
17 polynucleotides (Huber et al. *Anal. Biochem.* 212:351-358 (1993); US 5,585,236). The  
18 monolith was observed to have relatively large channels. Without wishing to be bound  
19 by theory, these channels are thought to allow rapid convective mass transport between  
20 the mobile phase and a thin outer layer of the polymer.

21 Applicants surprisingly discovered that the surface structure of the underivatized  
22 poly(styrene-divinylbenzene) monoliths resembled the surface structure of the octadecyl  
23 derivatized beads, both showing a surface that appeared rugulose, but not the  
24 underivatized beads, which showed a smooth surface. While derivatization with  
25 octadecyl groups has been shown to be essential to obtain high chromatographic  
26 efficiency with PS-DVB particles (Huber et al. *Anal. Biochem.* 212:351-358 (1993);  
27 Huber et al. *Nucleic Acids Res.* 21:1061-1066 (1993)) monolithic stationary phases  
28 exhibited superior efficiency already without derivatization. Without wishing to be bound  
29 by theory, one possible explanation for this different behavior is the formation of the  
30 polymer in two different chemical environments. The PS-DVB particles were  
31 polymerized in aqueous suspension, where poor solvation of the hydrophobic polymer  
32 by the hydrophilic solvent resulted in a relatively flat surface, as revealed by the  
33 scanning electron micrograph depicted in FIG. 7. Particles that were derivatized with  
34 octadecyl groups showed a rugulose surface (FIG. 8) possibly offering a contact area  
35 greater than that of a smooth spherical particle. The formation of the monolithic bed, on

1 the other hand, took place in an entirely organic environment. During polymerization,  
2 small primary particles of approximately 0.5  $\mu\text{m}$  coagulated to form the porous monolith,  
3 resulting in a surface structure (FIG. 9) that resembled the rugulose surface of the  
4 octadecylated PS-DVB particles (FIG. 8). Without wishing to be bound by theory,  
5 Applicants believe that the very rugulose surface of the stationary phase of the monolith  
6 of the present invention offers a contact area greater than that of smooth spherical  
7 particles and that this enhanced contact area gives improved separation performance. A  
8 "rugulose surface" as defined herein includes a surface characterized by showing many  
9 small wrinkles. It was also observed that a particle that was derivatized with octadecyl  
10 groups had a brush-like surface (FIG. 8). A "brush-like surface" as defined herein  
11 includes a surface characterized by showing many small bristles on the surface. The  
12 monolith (FIG. 9) also had a brush-like surface structure, unlike the underivatized  
13 particle (FIG. 7).

14 In still another aspect, the poly(styrene-divinylbenzene) monolith of the present  
15 invention provides a non-porous chromatographic surface. With a gradient of 4.0-  
16 12.0% acetonitrile in 50 mM TEAA in 10 min, oligothymidylic acids as small as the 3-  
17 mer were eluted as sharp and symmetric peaks (chromatogram not shown). From the  
18 crystal structure of the trinucleotide (A)<sub>3</sub> it can be inferred, that a 3-mer  
19 oligodeoxynucleotide has an almost globular structure with a diameter of approximately  
20 1.0 nm (Suck et al. *Acta Crystallogr., Sect. B* 32:1727-1737 (1976)). Because  
21 penetration of analytes into micropores of commensurate size would cause  
22 considerable band broadening, the capability of the monolithic stationary phase to  
23 efficiently separate such small molecules is a good indicator for the absence of  
24 micropores.

25 A still further aspect of the present invention is based on the surprising discovery  
26 that the underivatized poly(styrene-divinylbenzene) monoliths having nonpolar  
27 chromatographic surfaces were found to provide unusually high efficiency of separation  
28 of polynucleotides. In this aspect, the invention provides a monolith characterized by  
29 having high separation efficiency as indicated by a high number of theoretical plates per  
30 meter. Two terms are widely used as quantitative measures of band spreading and  
31 thus chromatographic column efficiency: the plate height  $H$  and the number of  
32 theoretical plates  $N$ . The two parameters are related by the equation:

$$N = L/H \quad (1)$$

The plate height and the dimensionless number of theoretical plates express the peak variance per unit length of the column and the dimensionless peak variance, respectively (Poole et al. *Chromatography Today*, Elsevier, Amsterdam (1995); *Practical HPLC Method Development* Snyder et al. Eds., John Wiley & Sons, New York, pp. 40-47 (1997)). Assuming that the form of the chromatographic peak can be approximated by a Gaussian curve, the number of theoretical plates can experimentally be determined from the equation:

$$N = 5.54 \left( \frac{t_R}{w_{0.5}} \right)^2 \quad (2)$$

$t_R$ ..... retention time [sec]

$w_{0.5}$ .... peak width at half height [sec]

The number of theoretical plates and the plate height are widely used in the art as measures of column performance. For these numbers to be meaningful in comparing two columns, it is essential that they are determined with the same compound and under the same isocratic elution conditions.

In a preferred embodiment of this aspect of the present invention, calculation of the number of theoretical plates is based on the retention time of a single polynucleotide standard under isocratic conditions. A preferred standard comprises a single-stranded oligodeoxynucleotide. In one example, the single-stranded polynucleotide, poly(dT)<sub>18</sub> was used as a standard for the determination of the number of theoretical plates per meter. The chromatographic efficiency of the monolithic columns was determined by isocratic elution of poly(dT)<sub>18</sub> with a mobile phase containing 7.8% acetonitrile in 100 mM TEAA at a flow rate of 2.4 µL/min. At 50°C column temperature, the number of theoretical plates exceeded 11,500 plates for a 60 mm column, corresponding to (N/L)= 191,000 theoretical plates per meter.

The capillary monolithic column of the present invention is characterized by having in the range of between about 10,000 and about 200,000 theoretical plates per meter, preferably between 100,000 and 200,000 theoretical plates per meter, more preferably at least 100,000 plates per meter, and most preferably at least 190,000 theoretical plates per meter. Without wishing to be bound by theory, it is believed that one of the main reasons for the high separation efficiency of the monoliths is the rapid mass transfer with the only particle-based diffusion limitation in a thin layer at the surface of monolith.

1 In another aspect, the invention provides a method for separating a mixture of  
2 polynucleotides in which the method includes applying the mixture of polynucleotides to  
3 a polymeric monolith, such as an underivatized poly(styrene-divinylbenzene) monolith,  
4 having non-polar chromatographic surfaces and eluting the mixture of polynucleotides  
5 with a mobile phase comprising a counterion agent and an organic solvent. When  
6 analyzing double-stranded polynucleotides, the method can be used to analyze  
7 polynucleotides having a wide range of lengths. For example, the method can be used  
8 in analyzing polynucleotides having lengths in the range of about 3 base pairs to about  
9 600 base pairs. The method can also be used in analyzing polynucleotides having up  
10 to about 2,000 base pairs. The elution step preferably uses a mobile phase containing  
11 a counterion agent and a water-soluble organic solvent. Examples of a suitable organic  
12 solvent include alcohol, acetonitrile, dimethylformamide, tetrahydrofuran, ester, ether,  
13 and mixtures of one or more thereof, e.g., methanol, ethanol, 2-propanol, 1-propanol,  
14 tetrahydrofuran, ethyl acetate, acetonitrile. The counterion agent is preferably selected  
15 from the group consisting of lower alkyl primary amine, lower alkyl secondary amine,  
16 lower alkyl tertiary amine, lower trialkylammonium salt, quaternary ammonium salt, and  
17 mixtures of one or more thereof. Examples of suitable counterion agents include  
18 triethylammonium acetate (TEAA) and triethylammonium bicarbonate (TEAB).

19 In an additional aspect, the invention provides a method for separating a mixture  
20 of polynucleotides in which the method includes applying the mixture of polynucleotides  
21 to a poly(styrene-divinylbenzene) monolith, such as underivatized poly(styrene-  
22 divinylbenzene), having non-polar chromatographic surfaces and eluting the mixture of  
23 polynucleotides with a mobile phase comprising a counterion agent and an organic  
24 solvent, wherein the mobile phase is devoid of metal chelating agent, such as EDTA.  
25 The elutions described in the Examples herein are performed using mobile phase  
26 lacking EDTA. Avoiding the use of EDTA is an advantage since EDTA in eluted  
27 fractions can interfere with subsequent mass spectral analysis. Removal of EDTA  
28 would require additional processing steps.

29 In a still further aspect, the invention concerns a method for separating a mixture  
30 of polynucleotides in which the method includes applying the mixture of polynucleotides  
31 to a poly(styrene-divinylbenzene) monolith having non-polar chromatographic surfaces  
32 and eluting the mixture of polynucleotides with a mobile phase comprising a counterion  
33 agent and an organic solvent, in which the method further includes analyzing the eluted  
34 polynucleotides by mass spectral analysis. The monolithic column can be operatively  
35 coupled to a mass spectrometer for determining the molecular mass of the eluted

1 polynucleotides. In a preferred embodiment, the mass spectrometer comprises an  
2 electrospray ionization (ESI) mass spectrometer. The electrospray ionization mass  
3 spectrometer can include a tandem mass spectrometer for determining the base  
4 sequences of the polynucleotides.

5 The possibility of direct on-line conjugation of capillary HPLC to mass  
6 spectrometry makes available highly valuable information about the structure and  
7 identity of the separated compounds (Tomer et al. *Mass Spectrom. Rev.* 13:431-457  
8 (1994)). Electrospray ionization mass spectrometry (ESI-MS), by virtue of the multiple  
9 charging of biopolymers and the very soft ionization process, has become one of the  
10 most important mass spectrometric techniques for the analysis of nucleic acids  
11 (Nordhoff et al. *P. Mass Spectrom. Rev.* 15:76-138 (1996)). Nevertheless, the success  
12 of ESI-MS for the characterization of nucleic acids largely depends on the purity of the  
13 sample that is introduced into the mass spectrometer (Portier et al. *Nucleic Acids Res.*  
14 22:3895-3903 (1994)). The major difficulties arise due to the tendency of nucleic acids  
15 to form quite stable adducts with cations resulting in mass spectra of poor quality (Stults  
16 et al. *Rapid Commun. Mass Spectrom.* 5:359-363 (1991); Huber et al. *Anal. Chem.*  
17 70:5288-5295 (1998)). As described hereinbelow, Applicants have observed that the  
18 on-line sample preparation of polynucleotides by chromatographic separation prior to  
19 ESI-MS removes cations from nucleic acid samples, and can be used to fractionate the  
20 polynucleotides in mixtures that are too complex for direct infusion ESI-MS.

21 The potential to obtain high quality ESI-mass spectra of large, double-stranded DNA is  
22 essentially determined by the amount of salt as well as the number of different  
23 compounds present in the sample mixture (Portier et al. *Nucleic Acids Res.* 22:3895-  
24 3903 (1994); Muddiman et al. *Anal. Chem.* 68:3705-3712 (1996)). Recently, Muddiman  
25 et. al. published the mass spectrum of a 500 bp polymerase chain reaction product,  
26 which has been purified by ethanol precipitation followed by microdialysis (Muddiman et  
27 al. *Rapid Commun. Mass Spectrom.* 13:1201-1204 (1999)). Although the amount of  
28 DNA that was analyzed in the ion cyclotron resonance mass spectrometer was in the  
29 low femtomol range, much more material was required for purification before mass  
30 measurement. Hence, there is an urgent need for rapid on-line separation and  
31 purification protocols requiring only minute sample amounts.

32 In a yet further aspect, the present invention provides a method for desalting and  
33 separating a mixture of single-stranded polynucleotides. The method includes dissolving  
34 a mixture of single-stranded polynucleotides in a mobile phase having a lower  
35 concentration of organic solvent than an initial mobile phase composition. The method

1 further includes loading the mixture onto a poly(styrene-divinylbenzene) monolithic  
2 column, as described herein, and flowing initial mobile phase containing a counterion  
3 agent and having a concentration of organic solvent that is below the level that would  
4 elute the polynucleotides through the column such that the polynucleotides are retained  
5 and the salts are removed from the polynucleotides. The method further includes  
6 separating the mixture of polynucleotides by eluting the mixture of polynucleotides with  
7 a mobile phase comprising a counterion agent and an organic solvent. This desalting  
8 method preferably includes preconcentrating the polynucleotides on the monolithic  
9 column. The volume loading capacity describes the maximum injection volume at  
10 constant analyte amount that can be loaded onto a separation column without the  
11 occurrence of peak broadening. Analytes which are present in extremely low  
12 concentrations in the sample may necessitate the injection of large sample volumes.  
13 Biomolecules exhibit very steep capacity curves in the reversed-phase mode and react  
14 very sensitive to small changes in mobile phase composition. Hence, a  
15 preconcentration at the column head occurs and injection of large volumes of sample  
16 containing a low concentration of analyte is possible without deleterious effects on the  
17 separation efficiency.

18 Monolithic capillary columns as described herein have numerous advantages  
19 when used in the separation of polynucleotides. The preparation can be carried out  
20 following simple procedures and an improved chromatographic separation performance  
21 can be obtained. Specific advantages include:

22 - The small volumes and low amounts of samples available from biochemical, medical  
23 or molecular biological experiments are most adequately processed by micro separation  
24 techniques.

25 - Polymerization within the confines of fused silica capillaries of small inner diameter is a  
26 straightforward way to manufacture monolithic columns for capillary and nano HPLC.

27 - By anchoring the chromatographic support material to the capillary wall using covalent  
28 chemical bonding, no tedious preparation of frits is necessary. Moreover, there is no  
29 need to pack columns using high pressure devices and no restrictions in achievable  
30 capillary length apply.

31 - The permeability of the monolithic capillary columns can be modulated by choosing an  
32 appropriate polymerization mixture. Columns with high permeability exhibit a lower back  
33 pressure than packed capillary columns and greater capillary lengths are possible for  
34 chromatographic separations.

- 1 - The enhanced mass transport through continuous macroporous polymer has a  
2 positive effect on chromatographic efficiency.  
3 - Expenses connected with consumption and disposal of materials are cut down.  
4 -The low flow rates applied in microcolumn high performance liquid chromatography are  
5 ideally suited for on-line coupling with electrospray ionization mass spectrometry.

6 Other features of the invention will become apparent in the course of the  
7 following descriptions of exemplary embodiments which are given for illustration of the  
8 invention and are not intended to be limiting thereof.

9 Procedures described in the past tense in the Examples below have been carried  
10 out in the laboratory. Procedures described in the present tense have not yet been  
11 carried out in the laboratory, and are constructively reduced to practice with the filing of  
12 this application.

#### 14 Example 1

##### 15 *Chemicals and oligodeoxynucleotide samples*

16 Acetonitrile (HPLC gradient-grade), divinylbenzene (synthesis grade), methanol  
17 (HPLC gradient-grade), styrene (synthesis grade), and tetrahydrofuran (analytical  
18 reagent grade) were obtained from Merck (Darmstadt, Germany). Styrene and  
19 divinylbenzene were distilled before use. Acetic acid (analytical reagent grade),  
20 azobisisobutyronitrile (synthesis grade), decanol (synthesis grade), and triethylamine  
21 (p.a.) were purchased from Fluka (Buchs, Switzerland). A 1.0 M stock solution of  
22 triethylammonium acetate (TEAA) was prepared by dissolving equimolar amounts of  
23 triethylamine and acetic acid in water. A 0.50 M stock solution of triethylammonium  
24 bicarbonate (TEAB) was prepared by passing carbon dioxide gas (AGA, Vienna,  
25 Austria) through a 0.50 M aqueous solution of triethylamine at 5°C until pH 8.4-8.9 was  
26 reached. For preparation of all aqueous solutions, high-purity water (Epure, Barnstead  
27 Co., Newton, MA, USA) was used. The standards of phosphorylated and non-  
28 phosphorylated oligodeoxynucleotides ((dT)<sub>12-18</sub>, p(dT)<sub>12-18</sub>, p(dT)<sub>19-24</sub>, p(dT)<sub>25-30</sub>) were  
29 purchased as sodium salts from Pharmacia (Uppsala, Sweden) or Sigma-Aldrich (St.  
30 Louis, MO, USA). The synthetic oligodeoxynucleotides (dT)<sub>24</sub> (M<sub>r</sub> 7,238.71), a 5'-  
31 dimethoxytritylated 5-mer (DMTr-ATGCG, M<sub>r</sub> 1805.42), and an 80-mer (M<sub>r</sub> 24,527.17):  
32 CCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTATCA  
33 GCAATAAACCAGCCAGCCGGAAGGG (SEQ ID NO:1)

1 were ordered from Microsynth (Balgach, Switzerland) and used without further  
2 purification. The size standard of double-stranded DNA restriction fragments (pBR322  
3 DNA-*Hae* III digest) was purchased from Sigma Aldrich.

#### 4 Example 2

##### 5 *Preparation of fused silica capillaries*

6 Fused silica capillaries with an inner diameter of 200  $\mu\text{m}$  and a length of 3 m were  
7 flushed with 2 mL of methanol and 2 mL of water, filled with 1 mol/L sodium hydroxide,  
8 closed at the ends and allowed to stand for 10 min at room temperature. Subsequently,  
9 the capillary was washed with 2 mL of water and 2 mL of methanol, and dried with  
10 nitrogen for 15 min at room temperature. Before *in situ* polymerization the inner wall of  
11 the fused silica tube was silanized in order to facilitate wetting by the solution of the  
12 monomer mixture and to allow covalent immobilization of the monolith in the tube  
13 (FIG.1). By attaching the bed to the tubing wall, gap formation between the capillary wall  
14 and the polymer due to shrinking of the polymer upon a change of solvent is avoided  
15 and no frit to support the bed is required.

16 In the silanization process, a mixture of 50% (v/v) 3-(trimethoxysilyl)propyl  
17 methacrylate and 0.01% (w/v) 2,2-diphenyl-1-picrylhydrazyl hydrate in  
18 dimethylformamide (DMF) was degassed with nitrogen for 5 min and filled into a  
19 pretreated, 3 m piece of fused silica capillary tubing (Huang et al. *J. Chromatogr. A*  
20 788:155-164 (1997)). The ends of the tubing were closed with silicon stoppers and the  
21 capillary was kept in an oven at 120°C for six hours. Next the capillary was flushed with  
22 2 mL each of DMF, methanol and dichloromethane, and finally dried with nitrogen.

#### 23 Example 3

##### 24 *Preparation of continuous-bed and packed-bed capillary columns*

25 Polyimide coated fused silica capillary tubing of 350  $\mu\text{m}$  OD and 200  $\mu\text{m}$  ID was  
26 obtained from Polymicro Technologies (Phoenix, AZ, USA). A 1 m piece of fused silica  
27 capillary tubing was silanized with 3-(trimethoxysilyl)propyl methacrylate (Huang et al.  
28 *C. J. Chromatogr. A* 788:155-164 (1997)) in order to ensure immobilization of the  
29 monolith at the capillary wall. Then, a 300 mm piece of the silanized capillary was filled  
30 with a mixture comprising 50  $\mu\text{L}$  styrene, 50  $\mu\text{L}$  divinylbenzene, 130  $\mu\text{L}$  decanol, 20  $\mu\text{L}$   
31 tetrahydrofuran, and 10 mg/mL azobisisobutyronitrile with a plastic syringe. The mixture  
32 was polymerized at 70 °C for 24 hours. After polymerization, the capillary was  
33 extensively flushed with acetonitrile at a flow rate of 5.0  $\mu\text{L}/\text{min}$  and finally cut into 60  
34 mm long pieces. Octadecylated PS-DVB particles (PS-DVB-C<sub>18</sub>) were synthesized as

published in the literature (Huber et al. *Anal. Biochem.* 212:351-358 (1993)). The PS-DVB-C<sub>18</sub> stationary phase has been commercialized as DNASep<sup>®</sup> columns by Transgenomic Inc. (San Jose, CA, USA). Packed-bed capillary columns were prepared according to the procedure described (Oberacher et al. *J. Chromatogr. A* (2000)).

#### Example 4

##### *High-performance liquid chromatography*

The HPLC system consisted of a low-pressure gradient micro pump (model Rheos 2000, Flux Instruments, Karlskoga, Sweden) controlled by a personal computer, a vacuum degasser (Knauer, Berlin, Germany), a column thermostat made from 3.3 mm OD copper tubing which was heated by means of a circulating water bath (model K 20 KP, Lauda, Lauda-Königshofen, Germany), a microinjector (model C4-1004, Valco Instruments Co. Inc., Houston, TX, USA) with a 200 or 500 nL internal sample loop, a variable wavelength detector (model UltiMate UV detector, LC Packings, Amsterdam, Netherlands) with a Z-shaped capillary detector cell (part no. ULT-UC-N-10, 3nL cell, LC Packings), and a PC-based data system (Chromeleon 4.30, Dionex-Softtron, Germering, Germany).

#### Example 5

##### *High-resolution capillary IP-RP-HPLC separation of phosphorylated oligodeoxynucleotide ladders in a monolithic capillary column*

Using the column as described in Example 3, a high-resolution capillary IP-RP-HPLC separation of a phosphorylated oligodeoxynucleotide ladder was performed (FIG. 2): Column, continuous PS-DVB, 80 × 0.20 mm ID; mobile phase, buffer A 100 mM TEAA, pH 6.80, buffer B 100 mM TEAA, pH 6.80, 20% acetonitrile; linear gradient, 32-42% B in 3.0 min, 42-52% B in 7 min; flow-rate, 3.3 µL/min; temperature, 50 °C; detection, UV, 254 nm; sample, p(dT)<sub>12-30</sub>, 6 ngram each/0.66 - 1.64 pmol each.

#### Example 6

##### *Separation of phosphorylated oligodeoxyadenylic- and oligothymidylic acids*

FIG. 3 illustrates the high-resolution separation of phosphorylated oligodeoxyadenylic- and oligothymidylic acids ranging in size from 12-30 nt. Gradient elution with 3.0-9.0% acetonitrile in 3.5 min, followed by 9.0-11.0% acetonitrile in 2.5, and finally 11.0-13.0% acetonitrile in 4.0 min in 100 mM TEAA resulted in peak widths at half height of 1.3 s for p(dA)<sub>12</sub> to 2.4 s for p(dT)<sub>30</sub> which allowed the baseline resolution of the whole series up to the 30-mer within 8.2 min. The resolution of homologous oligodeoxynucleotides obtained with the monolithic column clearly

surpasses that of a capillary column packed with PS-DVB-C<sub>18</sub> beads (Table 2, compare also Figure 1 in Huber et al. *Anal. Chem.* 71:3730-3739 (1999)).

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**Table 2**

**Comparison of the Resolution Values for Oligodeoxynucleotides and Double-stranded DNA using Packed and Monolithic Capillary Columns**

| compounds                                | resolution with packed column | resolution with monolithic column |
|------------------------------------------|-------------------------------|-----------------------------------|
| p(dT) <sub>12</sub> /p(dT) <sub>13</sub> | 3.05                          | 5.38                              |
| p(dT) <sub>29</sub> /p(dT) <sub>30</sub> | 1.04                          | 2.38                              |
| 51/57 bp                                 | 3.88                          | 5.15                              |
| 540/587 bp                               | 1.11                          | 2.70                              |

---

In this example the high-resolution capillary IP-RP-HPLC separation of phosphorylated oligodeoxynucleotide ladders was performed using a monolithic capillary column: Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A included 100 mM TEAA, pH 6.97, buffer B included 100 mM TEAA, pH 6.97, 20% acetonitrile; linear gradient, 15-45% B in 3.5 min, 45-55 % B in 2.5 min, 55-65 % B in 4.0 min; flow-rate, 2.5 µL/min; temperature, 50 °C; detection, UV, 254 nm; sample, p(dA)<sub>12-18</sub>, p(dT)<sub>12-30</sub>, 40 - 98 fmol of each oligodeoxynucleotide.

**Example 7**

*Performance of monolithic capillary columns for polynucleotide separations*

Following polymerization, extensive washing with acetonitrile, and equilibration with 100 mM TEAA-5.0% acetonitrile solution, the performance of three different 60 x 0.20 mm ID monolithic capillary columns was compared to that of three columns packed with octadecylated, 2.3 µm micropellicular PS-DVB particles of the same dimensions. The permeabilities of the monolithic columns and the packed columns were similar resulting in back pressures between 180 and 200 bar at a flow rate of 2.6 µL/min and 50°C column temperature, which indicates that the size of the channels for convective flow in both chromatographic beds is of approximately the same size. The relative standard deviations of the peak widths at half height both among various batches of packed capillary columns and monolithic capillary columns were better than 10% which demonstrates that column preparation was reproducible and allowed the comparison of the chromatographic performance of both column types. The chromatographic performance was evaluated by gradient separation of a mixture of (dT)<sub>12-18</sub> with a

1 gradient of 5.0-12.0% acetonitrile in 100 mM TEAA in 10 min. Three injections of the  
2 standard onto each of the three columns gave average peak widths at half height for  
3  $(dT)_{18}$  of  $2.28 \pm 0.22$  s (sample size  $N=9$ , standard deviation  $sd=0.29$  s, level of  
4 significance  $P=95\%$ ) for the monolithic columns and  $3.84 \pm 0.16$  s ( $N=9$ ,  $sd=0.20$  s,  
5  $P=95\%$ ) for the packed bed capillary column. These values demonstrate that the  
6 chromatographic performance of monolithic columns for oligodeoxynucleotide  
7 separations is approximately 40% better than that of packed bed columns. The  
8 chromatographic efficiency of the monolithic columns was determined by isocratic  
9 elution of  $(dT)_{18}$  with an eluent containing 7.8% acetonitrile in 100 mM TEAA at a flow  
10 rate of  $2.4 \mu\text{L}/\text{min}$ . At  $50^\circ\text{C}$  column temperature, the number of theoretical plates  
11 exceeded 11,500 plates for a 60 mm column, corresponding to 191,000 theoretical  
12 plates per meter.

#### 13 Example 8

##### 14 *High-resolution capillary IP-RP-HPLC separation of a mixture of phosphorylated and* 15 *dephosphorylated deoxyadenylic acids*

16 The separation shown in FIG. 4 was performed under the following condition:  
17 Column, monolithic PS-DVB, 60 mm x 0.20 mm ID; mobile phase, buffer A included 100  
18 mM TEAA, pH 7.00, buffer B included 100 mM TEAA, pH 7.00, 20% acetonitrile; linear  
19 gradient, 5-30% B in 5.0 min, 35-40 % B in 5.0 min, 40-45 % B in 6.0 min, 45-52 % B in  
20 14 min; flow-rate,  $2.1 \mu\text{L}/\text{min}$ ; temperature,  $50^\circ\text{C}$ ; detection, UV, 254 nm; sample,  
21 hydrolyzed  $p(dA)_{40}$  -  $p(dA)_{60}$ , spiked with 2.5 ng  $p(dA)_{12}$  -  $p(dA)_{18}$ .

#### 22 Example 9

##### 23 *Separation of double-stranded DNA using a PS-DVB monolithic column*

24 IP-RP-HPLC has been shown to be efficient not only for the rapid separation of  
25 single-stranded oligodeoxynucleotides, but also for the fractionation of double-stranded  
26 DNA fragments up to chain lengths of 2000 bp (Huber et al. *Anal. Chem.* 67:578-585  
27 (1995)). The applicability of the monolithic PS-DVB stationary phase to the IP-RP-HPLC  
28 separation of double-stranded DNA was tested by injection of a pBR322 DNA-*Hae* III  
29 digest, which was separated in 12.5 min using a gradient of 7.0-15.0% acetonitrile in 3  
30 min, followed by 15.0-19.0% acetonitrile in 12 min in 100 mM TEAA at a flow rate of  $2.2$   
31  $\mu\text{L}/\text{min}$  (FIG. 6). Again, the chromatogram of the mixture depicted in FIG. 6 with  
32 fragments ranging from 51-587 bp as well as the resolution values given in Table 2  
33 demonstrate that the separation performance of monolithic columns is superior to that of

1 packed-bed columns with respect to their separation capability for nucleic acids  
2 (compare also Figure 1 in Huber et al. *Anal. Chem.* 67:578-585 (1995)).

3 The separation shown in FIG. 5 was performed under the following conditions:  
4 Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 100 mM TEAA,  
5 pH 7.00, buffer B 100 mM TEAA, pH 7.00, 20% acetonitrile; linear gradient, 37-67% B in  
6 3.0 min, 67-87 % B in 7.0 min; flow-rate, 3.1 µL/min; temperature, 50°C; detection, UV,  
7 254 nm; sample, pBR322 DNA-*Hae* III digest, 12.1 ng, 4.5 fmol of each fragment.

8 The separation shown in FIG. 6 was performed under the following conditions:  
9 Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 100 mM TEAA,  
10 pH 7.00, buffer B 100 mM TEAA, pH 7.00, 20% acetonitrile; linear gradient, 35-75% B in  
11 3.0 min, 75-95 % B in 12.0 min; flow-rate, 2.2 µL/min; temperature, 50°C; detection, UV,  
12 254 nm; sample, pBR322 DNA-*Hae* III digest, 1.81 fmol of each fragment.

### 13 Example 10

#### 14 *Electrospray ionization mass spectrometry and coupling with capillary liquid* 15 *chromatography*

16 ESI-MS was performed on a Finnigan MAT LCQ quadrupole ion trap mass  
17 spectrometer (Finnigan MAT, San Jose, CA, USA, used in FIGs. 10-17) or a Finnigan  
18 MAT TSQ 7000 triple quadrupole mass spectrometer (used in FIGs. 5 and 6) equipped  
19 with an electrospray ion source. The capillary column was directly connected to the  
20 spray capillary (fused silica, 105 µm OD, 40 µm ID, Polymicro Technologies) by means  
21 of a microtight union (Upchurch Scientific, Oak Harbor, WA, USA). A syringe pump  
22 equipped with a 250 µL glass syringe (Unimetrics, Shorewood, IL, USA) was used for  
23 continuous infusion experiments and for pumping sheath liquid. For analysis with  
24 pneumatically assisted ESI, an electrospray voltage of 3.2-3.7 kV and a nitrogen sheath  
25 gas flow of 20-30 arbitrary units (LCQ) or 28-33 psi (TSQ) were employed. The  
26 temperature of the heated capillary was set to 200°C. Total ion chromatograms and  
27 mass spectra were recorded on a personal computer with the LCQ Navigator software  
28 version 1.2 or on a DEC-Alpha 3000 workstation with the ICIS software version 8.3.0  
29 (Finnigan). Mass calibration and coarse tuning was performed in the positive ion mode  
30 by direct infusion of a solution of caffeine (Sigma, St. Louis, MO, USA), methionyl-  
31 arginyl-phenylalanyl-alanine (Finnigan), and Ultramark 1621 (Finnigan). Fine tuning for  
32 ESI-MS of oligodeoxynucleotides in the negative ion mode was performed by infusion of  
33 3.0 µL/min of a 20 pmol/µL solution of (dT)<sub>24</sub> in 25 mM aqueous TEAB containing 10%  
34 acetonitrile (v/v). A sheath flow of 3.0 µL/min acetonitrile was added through the triaxial  
35 electrospray probe. For all direct infusion experiments, cations present in the

1 oligodeoxynucleotide samples were removed by on-line cation-exchange using a 20 ×  
2 0.50 mm ID cation-exchange microcolumn packed with 38-75 µm Dowex 50 WX8  
3 particles (Serva, Heidelberg, Germany) (Huber et al. M. R. *Anal. Chem.* 70:5288-5295  
4 (1998)). For IP-RP-HPLC-ESI-MS analysis, oligodeoxynucleotides and DNA fragments  
5 were injected without prior cation removal.

#### 6 Example 11

##### 7 *On-line separation and mass determination of synthetic oligodeoxynucleotides*

8 For many of the analytical problems encountered with oligodeoxynucleotides,  
9 chromatographic separation in combination with UV detection is not sufficient to get a  
10 conclusive answer. The on-line conjugation of chromatographic separation to mass  
11 spectrometry, however, offers a potent tool for the characterization and identification of  
12 oligodeoxynucleotides on the basis of accurate mass determinations and fragmentation  
13 patterns. For example, the HPLC-UV analysis of a (dT)<sub>12-18</sub> standard that was left  
14 overnight at room temperature showed a number of small peaks eluting before the  
15 seven major peaks (chromatogram not shown). Applicants supposed that the small  
16 peaks were phosphorylated or non-phosphorylated hydrolysis products of (dT)<sub>12-18</sub>, but  
17 this assumption was not definitive until the separation system was on-line coupled to  
18 ESI-MS, which revealed that they were non-phosphorylated hydrolyzates ranging from  
19 the 6-mer to the 11-mer (FIG. 10). Application of a gradient from 4.0-12.0% acetonitrile  
20 in 10 mM TEAA enabled the separation of all oligothymidylic acids from the 6-mer to the  
21 18-mer. Acetonitrile was added post-column as sheath liquid to enhance the mass  
22 spectrometric detectability of the separated oligodeoxynucleotides (Huber et al. *J.*  
23 *Chromatogr. A* 870:413-424 (2000)). This example demonstrates that by using on-line  
24 IP-RP-HPLC-ESI-MS, the unequivocal identification of low femtomol amounts of  
25 oligodeoxynucleotides is feasible on the basis of their molecular masses (Table 3).

Table 3

Measured and Theoretical Masses of (dT)<sub>6-18</sub>

| oligodeoxynucleotide | retention time<br>(min) | molecular mass |             | relative deviation<br>(%) |
|----------------------|-------------------------|----------------|-------------|---------------------------|
|                      |                         | measured       | theoretical |                           |
| (dT) <sub>6</sub>    | 1.77                    | 1763.09        | 1763.21     | 0.006                     |
| (dT) <sub>7</sub>    | 2.63                    | 2066.96        | 2067.40     | 0.021                     |
| (dT) <sub>8</sub>    | 3.59                    | 2371.90        | 2371.59     | -0.013                    |
| (dT) <sub>9</sub>    | 4.35                    | 2675.28        | 2675.79     | 0.019                     |
| (dT) <sub>10</sub>   | 4.94                    | 2978.95        | 2979.98     | 0.035                     |
| (dT) <sub>11</sub>   | 5.44                    | 3284.43        | 3284.18     | -0.008                    |
| (dT) <sub>12</sub>   | 5.76                    | 3589.29        | 3588.37     | -0.026                    |
| (dT) <sub>13</sub>   | 6.13                    | 3892.78        | 3892.57     | -0.006                    |
| (dT) <sub>14</sub>   | 6.39                    | 4197.47        | 4196.76     | -0.017                    |
| (dT) <sub>15</sub>   | 6.66                    | 4501.81        | 4500.96     | -0.019                    |
| (dT) <sub>16</sub>   | 6.92                    | 4806.26        | 4805.15     | -0.023                    |
| (dT) <sub>17</sub>   | 7.12                    | 5109.19        | 5109.35     | 0.003                     |
| (dT) <sub>18</sub>   | 7.35                    | 5413.35        | 5413.54     | 0.004                     |

The separation shown in FIG. 10 was performed under the following conditions:

Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 10 mM TEAA, pH 7.00, buffer B 10 mM TEAA, pH 7.00, 20% acetonitrile; linear gradient, 20-60% B in 10.0 min; flow-rate, 3.2 µL/min; temperature, 50 °C; scan, 800-2000 amu in 2 s; electrospray voltage, 3.8 kV; sheath gas, 34 psi N<sub>2</sub>; sheath liquid, acetonitrile; flow rate, 3.0 µL/min; sample, (dT)<sub>6-18</sub>, 50 ng.

## Example 12

*On-line coupling of chromatographic separation to mass spectrometry*

Refined chemistry has significantly improved the efficiency of automated solid-phase synthesis of long oligodeoxynucleotide sequences. However, assuming a coupling efficiency of 98-99% per synthesis cycle, the maximum yield of an 80-mer oligodeoxynucleotide will be only 20-45%, and contamination of the target sequence with a number of failure sequences or partially deprotected sequences is generally observed (Huber et al. *Anal. Chem.* 71:3730-3739 (1999); Huber et al. *LC GC Int.* 14:114-127 (1996)).<sup>28,40</sup> FIG. 11 illustrates the analysis of 5.0 pmol of a crude 80-mer oligodeoxynucleotide. The high number of partly resolved peaks eluting between 2 and

6 min made identification and quantitation of the target sequence from the reconstructed ion chromatogram impossible. However, extraction of a selected ion chromatogram at  $m/z$  1167.0, 1225.5, and 1290.0 clearly identified the target sequence eluting at 3.8 min (FIG. 12). Averaging and deconvolution of four mass spectra between 3.7 and 3.8 min yielded a molecular mass of 24,525.0 (FIG. 13 which correlates well with a theoretical mass of 24,527.17 (0.009% relative deviation). Moreover, the deconvoluted mass spectrum (FIG. 13) did not show notable cation adduction which verifies that IP-RP-HPLC is an efficient method for the desalting of oligodeoxynucleotides. Comparison of the mass spectrum extracted from the chromatogram (FIG. 13) to that of an 80-mer obtained by direct infusion ESI-MS (compare Figure 3 in Huber et al. *Anal. Chem.* 70:5288-5295 (1998)) clearly corroborates the high value of on-line coupling of chromatographic separation to mass spectrometry, because the chemical background in the mass spectrum is greatly reduced upon chromatographic separation and exact mass measurement is possible using IP-RP-HPLC-ESI-MS with only one fiftieth of the amount of sample that is consumed during direct infusion ESI-MS.

The separations shown in FIGs. 11-13 were performed under the following conditions: Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 25 mM TEAB, pH 8.40, buffer B 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient, 20-100% B in 15 min; flow-rate, 3.0  $\mu$ L/min; temperature, 50 °C; scan, 1000-3000 amu; electrospray voltage, 3.2 kV; sheath gas, 30 units; sheath liquid, acetonitrile; flow rate, 3.0  $\mu$ L/min; sample, 5.0 pmol raw product.

### Example 13

#### *On-line separation and mass determination of dsDNA fragments*

FIG. 14 illustrates the chromatogram of DNA fragments from 486 ng (180 fmol) of a pBR322 DNA-*Hae* III restriction digest with detection by ESI-MS. For this separation, the gradient was ramped from 3.0-6.0% acetonitrile in 3.0 min, followed by 6.0-10.0% acetonitrile in 12 min at a flow rate of 2.8  $\mu$ L/min and a column temperature of 40°C. The elution conditions for the spectra shown in FIGs. 14-17 were as follows: Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 25 mM TEAB, pH 8.40, buffer B 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient, 15-30% B in 3.0 min, followed by 30-50% B in 12 min; flow-rate, 2.8  $\mu$ L/min; temperature, 40°C; scan, 1000-3000 amu; electrospray voltage, 3.2 kV; sheath gas, 32 units; sheath liquid, acetonitrile; flow rate, 3  $\mu$ L/min; sample, pBR322 DNA-*Hae* III digest, 180 fmol of each fragment.

1 It can be seen that the fragments from 51-123 bp were completely resolved in the  
2 chromatogram, whereas the separation of the longer fragments was incomplete due to  
3 overloading of the column (Oberacher, H.; Krajete, A.; Parson, W.; Huber, C. G. J.  
4 *Chromatogr. A submitted (2000)*). Mass spectra were extracted from the reconstructed  
5 ion chromatogram by averaging 4-8 scans and three examples for fragments ranging in  
6 size from 80 to 267 bp are illustrated in FIGs. 15-17. Whereas relatively few charge  
7 states (23- - 29-) were found in the mass spectrum of an 80 bp fragment (FIG. 15), the  
8 number of observed signals rapidly increased with the size of the DNA fragments (FIGs.  
9 16 and 17). The appearance of all charge state signals with sharp and defined peak  
10 shapes indicates, that cation adducts have been efficiently removed by IP-RP-HPLC.

11 The molecular mass of the DNA fragments was calculated by a three step  
12 procedure. First, a rough molecular mass was obtained by automatic deconvolution of  
13 the raw spectrum using the Bioworks software application. For the fragments from 51-  
14 267 bp this deconvolution step readily yielded definite mass information and even the  
15 mass spectrum of the coeluting 123 bp and 124 bp fragments was easily deconvoluted  
16 into two separate mass peaks. For the longer DNA fragments (434-587 bp), signals for  
17 the individual charge states could be only identified using the knowledge of the  
18 theoretical molecular mass of the investigated fragments from their DNA sequence.  
19 Subsequently, the charge states of all  $m/z$  signals in the mass spectrum having an  
20 abundance more than five times the signal-to-noise ratio were calculated. Finally, the  
21  $m/z$  values and the corresponding integer charges state were used to calculate a  
22 molecular mass. Statistical treatment of the molecular masses of the individual charge  
23 states gave the average molecular mass and its standard deviation. The results of these  
24 calculations are summarized in (Table 4), which shows that the masses of the double-  
25 stranded DNA fragments ranging in size up to 267 bp were measured with an accuracy  
26 of better than 0.08%.

Table 4

**Molecular Masses of Double-stranded DNA Fragments from the pBR322 DNA-*Hae* III Digest**

| fragment | position <sup>a)</sup> | molecular mass         |             | relative deviation (%) |
|----------|------------------------|------------------------|-------------|------------------------|
|          |                        | measured <sup>b)</sup> | theoretical |                        |
| 51       | 942-992                | 31,565±24 (4)          | 31,559.57   | 0.018                  |
| 57       | 993-1049               | 35,252±54 (6)          | 35,263.04   | -0.032                 |
| 64       | 534-597                | 39,573±84 (7)          | 39,592.83   | -0.026                 |
| 80       | 3410-3489              | 49,494±43 (10)         | 49,475.35   | 0.038                  |
| 89       | 832-920                | 55,058±41 (14)         | 55,038.97   | 0.034                  |
| 104      | 298-401                | 64,391±56 (22)         | 64,312.99   | 0.12                   |
| 123      | 175-297                | 76,059±49 (15)         | 76,045.76   | 0.017                  |
| 124      | 402-525                | 76,731±44 (17)         | 76,675.05   | 0.073                  |
| 184      | 1263-1446              | 113,802±140 (15)       | 113,747.36  | 0.048                  |
| 192      | 4344-174               | 118,722±123 (17)       | 118,668.82  | 0.045                  |
| 213      | 1050-1262              | 131,733±148 (18)       | 131,674.02  | 0.045                  |
| 234      | 598-831                | 144,708±127 (25)       | 144,646.56  | 0.042                  |
| 267      | 3490-3756              | 165,091±230 (12)       | 165,019.11  | 0.044                  |
| 434      | 2518-2951              | n. d. <sup>c)</sup>    | 268,240.41  | n. d.                  |
| 458      | 2952-3409              | n. d.                  | 283,002.81  | n. d.                  |
| 502      | 1447-1948              | n. d.                  | 310,240.12  | n. d.                  |
| 540      | 1949-2488              | n. d.                  | 333,738.33  | n. d.                  |
| 587      | 3757-4343              | n. d.                  | 362,707.09  | n. d.                  |

<sup>a)</sup>position relative to the *EcoR* I restriction site in pBR322.

<sup>b)</sup>molecular mass given as average±standard deviation (number of charge states used to calculate the average molecular mass).

<sup>c)</sup>not determined.

**Example 14**

***IP-RP-HPLC-ESI-MS/MS sequencing of oligodeoxynucleotides***

In addition to information regarding the molecular mass, tandem mass spectrometry (MS/MS) utilizing collisionally induced dissociation (CID) provides valuable

1 information about the base sequence of oligodeoxynucleotides (McLuckey et al.  
2 *Tandem Mass Spectrometry of Small, Multiply Charged Oligodeoxynucleotides* 3:pp 60-  
3 70 (1992); Griffey et al. *J. Mass Spectrom.* 32:305-313 (1997)). In this example, for the  
4 application of monolithic capillary columns in nucleic acid analysis, the feasibility to  
5 perform on-line MS/MS experiments on oligodeoxynucleotides upon liquid  
6 chromatographic separation was examined. To evaluate the performance of IP-RP-  
7 HPLC-ESI-MS/MS for oligodeoxynucleotide sequencing, a 5-mer oligodeoxynucleotide  
8 (sequence 5'-ATGCG-3') was ordered from Microsynth. The IP-RP-HPLC-ESI-MS  
9 analysis of the unfragmented 5-mer gave a molecular mass of 1805.00, which  
10 exceeded the expected mass value of 1503.04 by 301.96 mass units. This mass  
11 difference could be attributable to an additional thymidine residue (probably entered into  
12 the synthesis automat by accident) or to a 5'-terminal dimethoxytrityl protecting group  
13 (that has been forgotten to hydrolyze after the last coupling cycle). Substantially  
14 increased retention in the chromatographic analysis was indicative for the latter  
15 assumption. The presence of a dimethoxytrityl protecting group as well as the total  
16 sequence of the oligodeoxynucleotide was confirmed using IP-RP-HPLC-ESI-MS/MS  
17 (FIGs. 18 and 19). The ESI-MS/MS experiment was performed by isolating the  $[M-2H]^{2-}$   
18 charge state at  $m/z$  901.37 and collisional activation at 19% relative collision energy.  
19 Assignments and masses for the fragment ions observed in the tandem mass spectrum  
20 (FIG. 19) are listed in Table 5.

21

Table 5

## Fragment Ions for Sequencing of a 5-mer Oligodeoxynucleotide

| Ion assignment                    | m/z     |
|-----------------------------------|---------|
| (M) <sup>2-</sup>                 | 901.37  |
| (M-A) <sup>2-</sup>               | 833.65  |
| (M-T) <sup>2-</sup>               | 838.57  |
| (M-G) <sup>2-</sup>               | 826.13  |
| (M-C) <sup>2-</sup>               | 845.89  |
| (w <sub>1</sub> ) <sup>1-</sup>   | 345.87  |
| (w <sub>2</sub> ) <sup>1-</sup>   | 635.06  |
| (w <sub>3</sub> ) <sup>1-</sup>   | 964.18  |
| (w <sub>4</sub> ) <sup>1-</sup>   | 1267.01 |
| (w <sub>3</sub> ) <sup>2-</sup>   | 481.45  |
| (w <sub>4</sub> ) <sup>2-</sup>   | 633.53  |
| (a <sub>2</sub> -T) <sup>1-</sup> | 714.16  |
| (a <sub>3</sub> -G) <sup>1-</sup> | 1016.07 |
| (a <sub>4</sub> -C) <sup>1-</sup> | 1345.98 |

Beside the parent ion all four ions that show loss of one nucleobase are observed. The most diagnostic ions however arise from fragmentation which produces w series ions, that are used to determine the 3'→5' sequence and the a<sub>n</sub>-B<sub>n</sub> series ions, that are used to determine the 5'→3' sequence (McLuckey et al. *Tandem Mass Spectrometry of Small, Multiply Charged Oligodeoxynucleotides* 3:60-70 (1992)). The complete w series is present in the MS/MS spectrum and the masses correspond to those expected for an oligodeoxynucleotide with the sequence 5'-ATGCG-3', proving that the 3' terminus is unmodified. The a<sub>n</sub>-B<sub>n</sub> series however shows a mass shift of +302 from the expected mass, corresponding to the presence of the dimethoxytrityl protecting group at the 5' terminus. Finally the presence of the protective group was confirmed by cleavage with 2% formic acid at room temperature for 5 minutes, yielding the oligodeoxynucleotide ATGCG with the expected mass of 1502.98.

The separations shown in FIGs. 18 and 19 were performed under the following conditions: Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 25 mM TEAB, pH 8.40, buffer B 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient,

1 10-100% B in 5.0 min; flow-rate, 3.0  $\mu$ L/min; temperature, 50 °C; daughter ions of m/z  
2 901.5, 4.0 amu isolation width, 19% relative collision energy; scan, 250-1810 amu;  
3 electrospray voltage, 3.2 kV; sheath gas, 30 units; sheath liquid, acetonitrile; flow rate,  
4 3.0  $\mu$ L/min; sample, 25 pmol raw product.

5

6 While the foregoing has presented specific embodiments of the present  
7 invention, it is to be understood that these embodiments have been presented by way of  
8 example only. It is expected that others will perceive and practice variations which,  
9 though differing from the foregoing, do not depart from the spirit and scope of the  
10 invention as described and claimed herein.

1 The invention claimed is:

- 2 1. A method for separating a mixture of polynucleotides, said method comprising:  
3 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
4 chromatographic surfaces and eluting said mixture of polynucleotides with a  
5 mobile phase comprising a counterion agent and an organic solvent,  
6 wherein said monolith is contained within a fused silica tube having an inner  
7 diameter in the range of 1 micrometer to 1000 micrometer,  
8 wherein said monolith is immobilized by covalent attachment at the inner wall of  
9 said tube, and  
10 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
11 matrix.
- 12 2. A method of claim 1 wherein said tube is devoid of retaining frits.
- 13 3. A method of claim 1 wherein said monolith is characterized by having 100,000 to  
14 200,000 theoretical plates per meter.
- 15 4. A method of claim 3 wherein said theoretical plates per meter is determined from the  
16 retention time of single stranded p(dT)<sub>18</sub> standard using the following equation:  
17

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

- 18 wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
19 standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half  
20 height, and  $L$  is the length of the monolith in meters.
- 21 5. A method of claim 4 wherein said tube has an inner diameter of 200 micrometer and  
22 a length of 60 mm, wherein during said isocratic elution said monolith has a back  
23 pressure in the range of 180 to 200 bar, and a flow rate in the range of 2 to 3  $\mu\text{L}/$   
24 min at an elution temperature of 50°C.
- 25 6. A method of claim 1 wherein said mobile phase is devoid of EDTA.
- 26 7. A method of claim 1 wherein said monolith has a surface morphology, as determined  
27 by scanning electron microscopy, that resembles the surface morphology of  
28 octadecyl modified poly(styrene-divinylbenzene) particles, wherein said surface  
29 morphology of said monolith is brush-like.
- 30 8. A method of claim 1 wherein said monolith has a surface morphology, as determined  
31 by scanning electron microscopy, that resembles the surface morphology of

1 octadecyl modified poly(styrene-divinylbenzene) particles, wherein said surface  
2 morphology of said monolith is rugulose.

3

4 9. A method for separating a mixture of polynucleotides, said method comprising:  
5 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
6 chromatographic surfaces and eluting said mixture of polynucleotides with a  
7 mobile phase comprising a counterion agent and an organic solvent,  
8 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
9 matrix,  
10 wherein said monolith is contained within a fused silica tube, and  
11 wherein said monolith is immobilized by covalent attachment at the inner wall of  
12 said tube.

13 10. A method of claim 9 wherein said monolith is contained within said fused silica tube  
14 having an inner diameter in the range of 1 micrometer to 1000 micrometer.

15 11. A method of claim 9 wherein said tube is devoid of retaining frits.

16 12. A method of claim 9 wherein said monolith is characterized by having 100,000 to  
17 200,000 theoretical plates per meter.

18 13. A method of claim 9 wherein said mobile phase is devoid of EDTA.

19 14. A method of claim 9 wherein said monolith has a surface morphology, as  
20 determined by scanning electron microscopy, that resembles the surface  
21 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
22 said surface morphology of said monolith is brush-like.

23 15. A method of claim 9 wherein said monolith has a surface morphology, as  
24 determined by scanning electron microscopy, that resembles the surface  
25 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
26 said surface morphology of said monolith is rugulose.

27

28 16. A method for separating a mixture of polynucleotides, said method comprising:  
29 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
30 chromatographic surfaces and eluting said mixture of polynucleotides with a  
31 mobile phase comprising a counterion agent and an organic solvent,  
32 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
33 matrix,  
34 wherein said monolith is contained within a fused silica tube,

- 1 wherein said tube has an inner diameter in the range of 1 micrometer to 1000  
2 micrometer,  
3 wherein said tube is devoid of retaining frits, and  
4 wherein said polynucleotides comprise double-stranded fragments having  
5 lengths in the range of 3 to 600 base pairs.
- 6 17. A method of claim 16 wherein said mobile phase is devoid of EDTA.
- 7 18. A method of claim 17 wherein said monolith is immobilized by covalent attachment  
8 at the inner wall of said tube.
- 9 19. A method of claim 16 wherein said monolith is characterized by having 100,000 to  
10 200,000 theoretical plates per meter.
- 11 20. A method of claim 16 wherein said monolith has a surface morphology, as  
12 determined by scanning electron microscopy, that resembles the surface  
13 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
14 said surface morphology of said monolith is brush-like.
- 15 21. A method of claim 16 wherein said monolith is characterized by having at least  
16 100,000 theoretical plates per meter.
- 17 22. A method of claim 16 wherein said monolith has a surface morphology, as  
18 determined by scanning electron microscopy, that resembles the surface  
19 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
20 said surface morphology of said monolith is rugulose.
- 21 23. A method for separating a mixture of polynucleotides, said method comprising:  
22 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
23 chromatographic surfaces and eluting said mixture of polynucleotides with a  
24 mobile phase comprising a counterion agent and an organic solvent,  
25 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
26 matrix,  
27 wherein said monolith is characterized by having 10,000 to 200,000 theoretical  
28 plates per meter,  
29 wherein said monolith is contained within a fused silica tube having an inner  
30 diameter in the range of 1 micrometer to 1000 micrometer, and  
31 wherein said monolith is immobilized by covalent attachment at the inner wall of  
32 said tube.
- 33 24. A method of claim 23 wherein said theoretical plates per meter is determined from  
34 the retention time of single stranded p(dT)<sub>18</sub> standard using the following  
35 equation:

1

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

2 wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
3 standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half  
4 height, and  $L$  is the length of the monolith in meters.

5 25. A method of claim 23 wherein said monolith has a surface morphology, as  
6 determined by scanning electron microscopy, that resembles the surface  
7 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
8 said surface morphology of said monolith is brush-like

9 26. A method of claim 23 wherein said tube is silianized.

10 27. A method of claim 23 wherein said tube is devoid of retaining frits.

11 28. A method of claim 23 wherein said mobile phase is devoid of EDTA.

12 29. A method of claim 23 wherein said monolith has a surface morphology, as  
13 determined by scanning electron microscopy, that resembles the surface  
14 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
15 said surface morphology of said monolith is rugulose.

16 30. A method for separating a mixture of polynucleotides, said method comprising:  
17 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
18 chromatographic surfaces and eluting said mixture of polynucleotides with a  
19 mobile phase comprising a counterion agent and an organic solvent,  
20 wherein said monolith is contained within a fused silica tube having an inner  
21 diameter in the range of 1 micrometer to 1000 micrometer,  
22 wherein said mobile phase is devoid of EDTA,  
23 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
24 matrix

25 31. A method of claim 30 wherein said monolith has a surface morphology, as  
26 determined by scanning electron microscopy, that resembles the surface  
27 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
28 said surface morphology of said monolith is brush-like.

29 32. A method of claim 30 wherein said monolith is immobilized by covalent attachment  
30 at the inner wall of said tube.

31 33. A method of claim 32 wherein said tube is devoid of retaining frits.

- 1 34. A method of claim 30 wherein said monolith is characterized by having 10,000 to  
2 200,000 theoretical plates per meter.
- 3 35. A method of claim 30 wherein said monolith has a surface morphology, as  
4 determined by scanning electron microscopy, that resembles the surface  
5 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
6 said surface morphology of said monolith is rugulose.
- 7 36. A method of claim 30 wherein said tube has been silanized.
- 8
- 9 37. A method for separating a mixture of polynucleotides, said method comprising:  
10 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
11 chromatographic surfaces and eluting said mixture of polynucleotides with a  
12 mobile phase comprising a counterion agent and an organic solvent,  
13 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
14 matrix,  
15 wherein said monolith has a surface morphology, as determined by scanning  
16 electron microscopy, that resembles the surface morphology of octadecyl  
17 modified poly(styrene-divinylbenzene) particles, wherein said surface  
18 morphology of said monolith is rugulose.
- 19 38. A method of claim 37 wherein said mobile phase is devoid of EDTA.
- 20 39. A method of claim 37 wherein said monolith is contained within a fused silica tube  
21 having an inner diameter in the range of 1 micrometer to 1000 micrometer.
- 22 40. A method of claim 37 wherein said monolith is immobilized by covalent attachment  
23 at the inner wall of said tube.
- 24 41. A method of claim 37 wherein said tube is devoid of retaining frits.
- 25 42. A method of claim 37 wherein said monolith is characterized by having 100,000 to  
26 200,000 theoretical plates per meter.
- 27 43. A method of claim 37 wherein said monolith has a surface morphology, as  
28 determined by scanning electron microscopy, that resembles the surface  
29 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
30 said surface morphology of said monolith is brush-like.
- 31
- 32 44. A method for separating a mixture of polynucleotides, said method comprising:  
33 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
34 chromatographic surfaces and eluting said mixture of polynucleotides with a  
35 mobile phase comprising a counterion agent and an organic solvent,

1 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
2 matrix,  
3 wherein said monolith is contained within a fused silica tube having an inner  
4 diameter in the range of 1 micrometer to 1000 micrometer,  
5 wherein said monolith is immobilized at the inner wall of said tube,  
6 wherein said tube is devoid of retaining frits.

7 45. A method of claim 44 wherein said mobile phase is devoid of EDTA.

8 46. A method of claim 44 wherein said monolith is contained within a tube having an  
9 inner diameter in the range of 10 micrometer to 300 micrometer.

10 47. A method of claim 44 wherein said monolith is immobilized at the inner wall of said  
11 tube and wherein said tube has been silanized.

12 48. A method of claim 44 wherein said monolith has a surface morphology, as  
13 determined by scanning electron microscopy, that resembles the surface  
14 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
15 said surface morphology of said monolith is brush-like.

16 49. A method of claim 44 wherein said monolith is characterized by having 100,000 to  
17 200,000 theoretical plates per meter.

18 50. A method of claim 44 wherein said monolith has a surface morphology, as  
19 determined by scanning electron microscopy, that resembles the surface  
20 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
21 said surface morphology of said monolith is rugulose.

22

23 51. A device for separating a mixture of polynucleotides, said device comprising:  
24 a polymeric monolith having non-polar chromatographic surfaces,  
25 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
26 matrix,  
27 wherein said monolith is contained within a fused silica tube having an inner  
28 diameter in the range of 1 micrometer to 1000 micrometer, wherein said monolith  
29 is immobilized by covalent attachment at the inner wall of said tube.

30 52. A device of claim 51 wherein said tube is devoid of retaining frits.

31 53. A device of claim 51 wherein said monolith is characterized by having 100,000 to  
32 200,000 theoretical plates per meter.

33 54. A device of claim 53 wherein said theoretical plates per meter is determined from  
34 the retention time of single stranded p(dT)<sub>18</sub> standard using the following  
35 equation:

1

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

2 wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
3 standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half  
4 height, and  $L$  is the length of the monolith in meters.

5 55. A device of claim 54 wherein said tube has an inner diameter of 200 micrometer  
6 and a length of 60 mm, wherein during said isocratic elution said monolith has a  
7 back pressure in the range of 180 to 200 bar, and a flow rate in the range of 2 to  
8 3  $\mu\text{L}/\text{min}$  at an elution temperature of 50°C.

9 56. A device of claim 51 wherein said monolith has a surface morphology, as  
10 determined by scanning electron microscopy, that resembles the surface  
11 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
12 said surface morphology of said monolith is rugulose.

13 57. A device of claim 51 wherein the chromatographic surfaces of said monolith are  
14 devoid of micropores.

15 58. A device of claim 57 wherein said monolith has channels sufficiently large for  
16 convective flow of said mobile phase.

17

18 59. A device for separating a mixture of polynucleotides, said device comprising:  
19 a polymeric monolith having non-polar chromatographic surfaces,  
20 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
21 matrix,  
22 wherein said monolith is contained within a fused silica tube, and  
23 wherein said monolith is immobilized by covalent attachment at the inner wall of  
24 said tube.

25 60. A device of claim 59 wherein said tube has an inner diameter in the range of 1  
26 micrometer to 1000 micrometer.

27 61. A device of claim 59 wherein said tube is devoid of retaining frits.

28 62. A device of claim 59 wherein said monolith is characterized by having 10,000 to  
29 200,000 theoretical plates per meter.

30 63. A device of claim 59 wherein said monolith has a surface morphology, as  
31 determined by scanning electron microscopy, that resembles the surface

1 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
2 said surface morphology of said monolith is brush-like.

3 64. A device of claim 59 wherein said monolith comprises an underivatized monolithic  
4 stationary phase.

5 65. A device of claim 59 wherein said monolith has a surface morphology, as  
6 determined by scanning electron microscopy, that resembles the surface  
7 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
8 said surface morphology of said monolith is rugulose.

9 66. A device of claim 59 wherein said monolith is devoid of micropores and wherein  
10 said monolith has channels sufficiently large for convective flow of said mobile  
11 phase.

12

13 67. A device for separating a mixture of polynucleotides, said device comprising:  
14 a polymeric monolith having non-polar chromatographic surfaces,  
15 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
16 matrix,  
17 wherein said monolith is contained within a fused silica tube,  
18 wherein said tube has been silanized, and  
19 wherein said tube is devoid of retaining frits.

20 68. A device of claim 67 wherein said monolith is immobilized by covalent attachment  
21 at the inner wall of said tube.

22 69. A device of claim 67 wherein said monolith is characterized by having 100,000 to  
23 200,000 theoretical plates per meter.

24 70. A device of claim 67 wherein said monolith has a surface morphology, as  
25 determined by scanning electron microscopy, that resembles the surface  
26 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
27 said surface morphology of said monolith is brush-like.

28 71. A device of claim 67 wherein said tube has an inner diameter in the range of 1  
29 micrometer to 1000 micrometer.

30 72. A device of claim 67 wherein said monolith has a surface morphology, as  
31 determined by scanning electron microscopy, that resembles the surface  
32 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
33 said surface morphology of said monolith is rugulose.

34

35 73. A device for separating a mixture of polynucleotides, said device comprising:

- 1 a polymeric monolith having non-polar chromatographic surfaces,  
2 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
3 matrix,  
4 wherein said monolith is contained within a tube having an inner diameter in the  
5 range of 1 micrometer to 1000 micrometer,  
6 wherein said monolith is characterized by having 10,000 to 200,000 theoretical  
7 plates per meter.
- 8 74. A device of claim 73 wherein said monolith is contained within a tube having an  
9 inner diameter in the range of 1 micrometer to 1000 micrometer.
- 10 75. A device of claim 73 wherein said monolith is immobilized by covalent attachment  
11 at the inner wall of said tube.
- 12 76. A device of claim 75 wherein said tube is devoid of retaining frits.
- 13 77. A method of claim 73 wherein said monolith has a surface morphology, as  
14 determined by scanning electron microscopy, that resembles the surface  
15 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
16 said surface morphology of said monolith is brush-like.
- 17 78. A method of claim 73 wherein said monolith has a surface morphology, as  
18 determined by scanning electron microscopy, that resembles the surface  
19 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
20 said surface morphology of said monolith is rugulose.
- 21
- 22 79. A device for separating a mixture of polynucleotides, said device comprising:  
23 a polymeric monolith having non-polar chromatographic surfaces,  
24 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
25 matrix,  
26 wherein said monolith is characterized by having at least 100,000 theoretical  
27 plates per meter,  
28 wherein said monolith is contained within a silanized fused silica tube having an  
29 inner diameter in the range of 10 micrometer to 1000 micrometer,  
30 wherein said monolith is immobilized at the inner wall of said tube.
- 31 80. A device of claim 79 wherein said monolith is characterized by having 100,000 to  
32 200,000 theoretical plates per meter.
- 33 81. A device of claim 79 wherein said monolith is contained within a tube having an  
34 inner diameter in the range of 1 micrometer to 1000 micrometer.

- 1 82. A device of claim 79 wherein said monolith has a surface morphology, as  
2 determined by scanning electron microscopy, that resembles the surface  
3 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
4 said surface morphology of said monolith is brush-like.
- 5 83. A device of claim 82 wherein said tube is devoid of retaining frits.
- 6 84. A device of claim 79 wherein said monolith has a surface morphology, as  
7 determined by scanning electron microscopy, that resembles the surface  
8 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
9 said surface morphology of said monolith is rugulose.
- 10 85. A miniaturized chromatographic system for separating a mixture of polynucleotides,  
11 said system comprising the device of claim 79.
- 12 86. A device for separating a mixture of polynucleotides, said device comprising:  
13 a polymeric monolith having non-polar chromatographic surfaces,  
14 wherein said monolith has a surface morphology, as determined by scanning  
15 electron microscopy, that resembles the surface morphology of octadecyl  
16 modified poly(styrene-divinylbenzene) particles, wherein said surface  
17 morphology of said monolith is rugulose and brush-like,  
18 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
19 matrix,  
20 wherein said monolith is contained within a fused silica tube having an inner  
21 diameter in the range of 1 micrometer to 1000 micrometer,  
22 wherein said monolith is immobilized at the inner wall of said tube.
- 23 87. A device of claim 86 wherein said tube is devoid of retaining frits.
- 24 88. A device of claim 86 wherein said monolith is characterized by having 100,000 to  
25 200,000 theoretical plates per meter.
- 26 89. A device of claim 86 wherein said tube has been silanized.
- 27 90. A device of claim 86 wherein said surfaces of said monolith are non-porous.
- 28 91. A device of claim 86 wherein said monolith is formed from a polymerization mixture  
29 including underivatized styrene, a crosslinking agent, and a porogen, wherein  
30 said porogen comprises tetrahydrofuran.
- 31 92. A device of claim 86 wherein said polynucleotides comprise double-stranded  
32 fragments having lengths in the range of 3 to 600 base pairs.
- 33 93. A method of claim 16 including analyzing eluted polynucleotides by mass spectral  
34 analysis.

- 1 94. A method of claim 23 including analyzing eluted polynucleotides by mass spectral  
2 analysis.
- 3 95. A system of claim 85 wherein said monolith is operatively coupled to a mass  
4 spectrometer.
- 5 96. A method for desalting a mixture of polynucleotides, said method comprising:  
6 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
7 chromatographic surfaces and eluting said mixture of polynucleotides with a  
8 mobile phase comprising a counterion agent and an organic solvent,  
9 wherein said monolith is characterized by having 100,000 to 200,000 theoretical  
10 plates per meter,  
11 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
12 matrix,  
13 wherein said monolith is contained within a fused silica tube having an inner  
14 diameter in the range of 1 micrometer to 1000 micrometer,  
15 wherein said monolith is immobilized at the inner wall of said tube.
- 16 97. A chromatographic device, said device comprising:  
17 a polymeric monolith having non-polar chromatographic surfaces,  
18 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
19 matrix,  
20 wherein said monolith is contained within a silanized fused silica tube having an  
21 inner diameter in the range of 10 micrometer to 1000 micrometer, and  
22 wherein said monolith is immobilized at the inner wall of said tube.

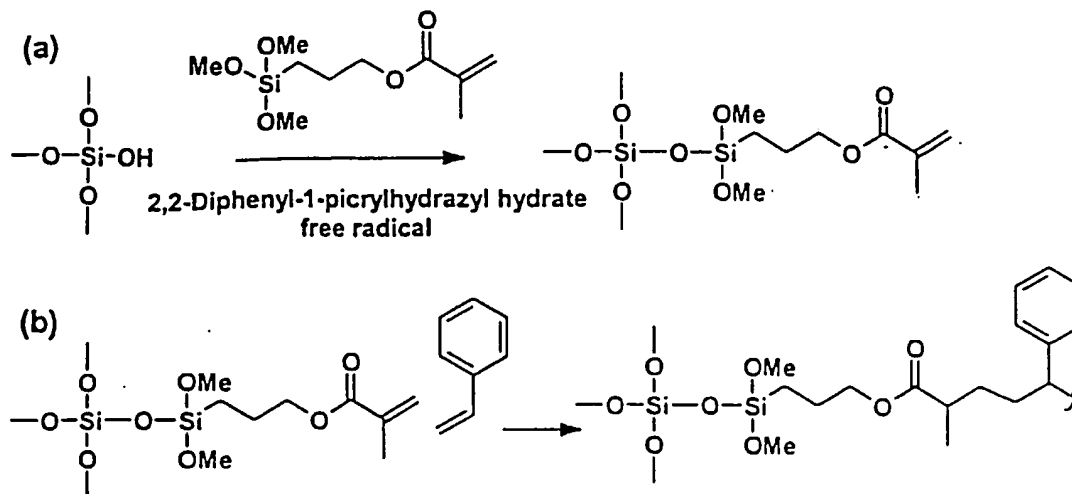


FIG. 1

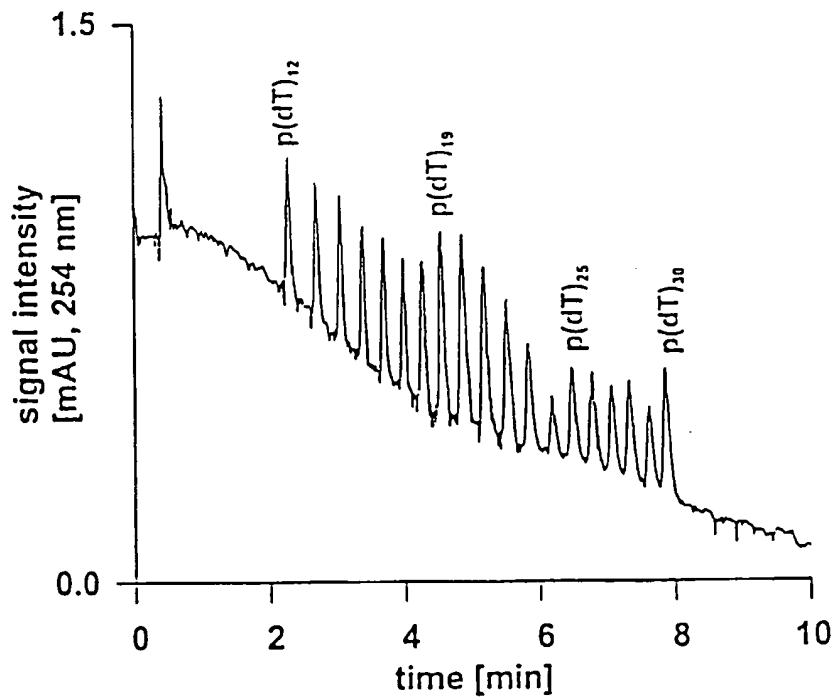


FIG. 2

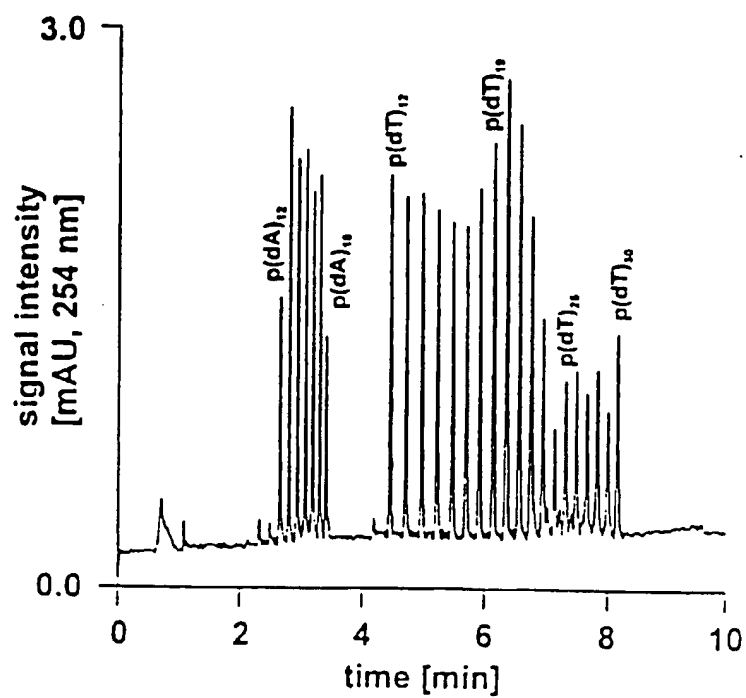


FIG. 3

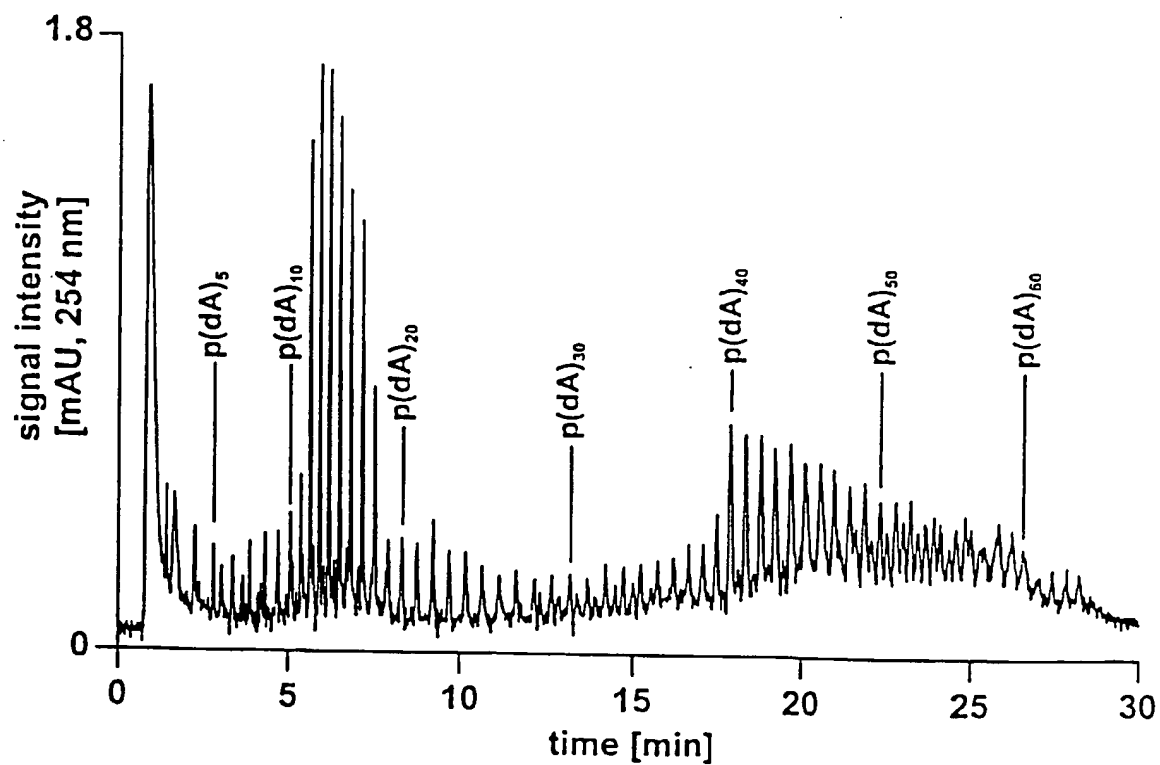


FIG. 4

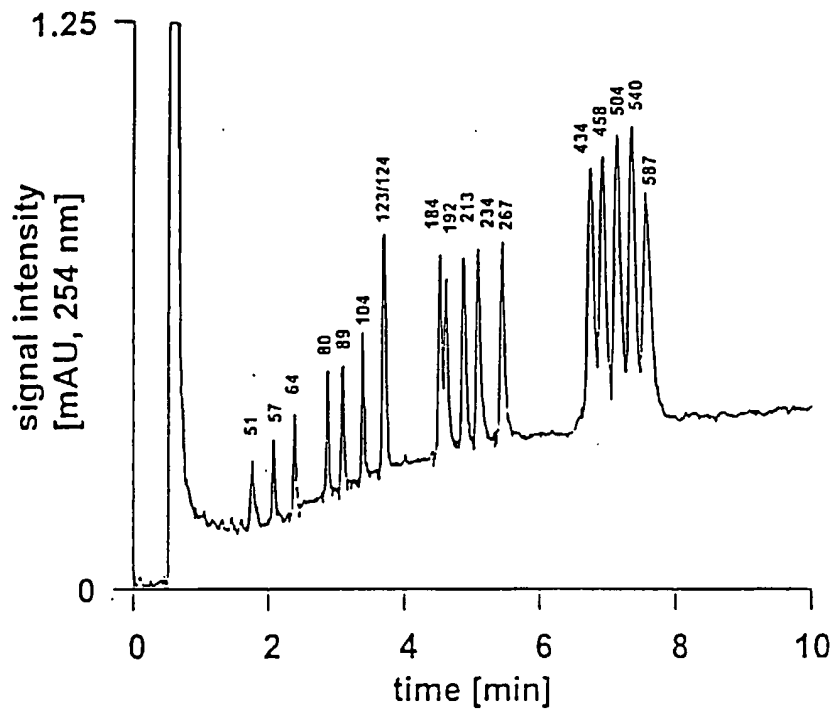


FIG. 5

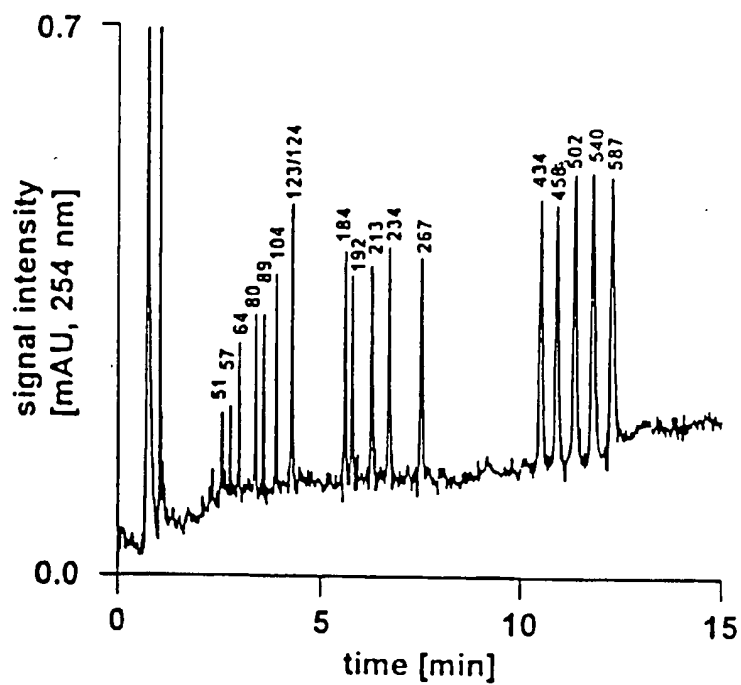
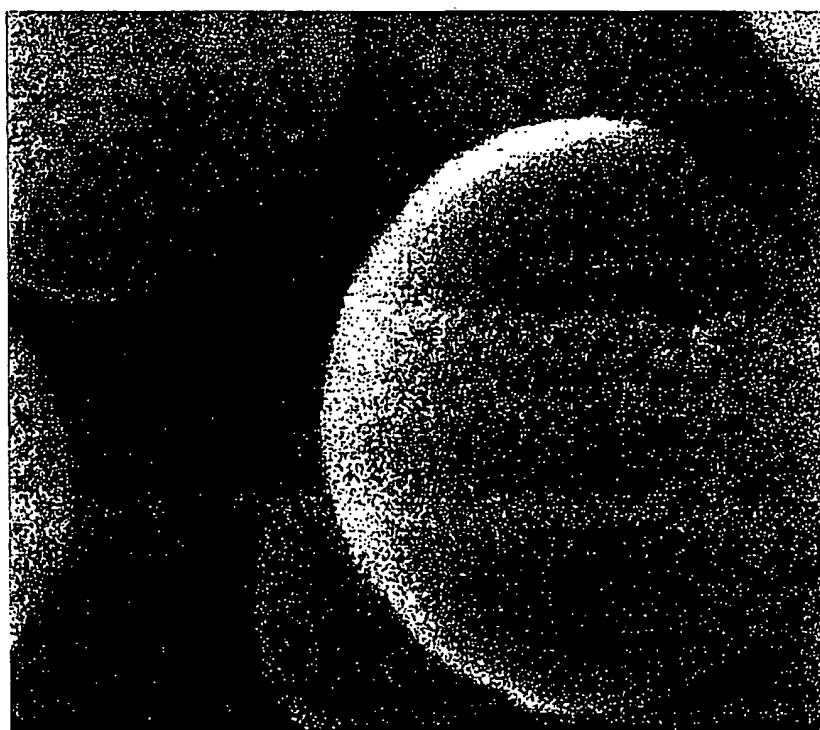


FIG. 6



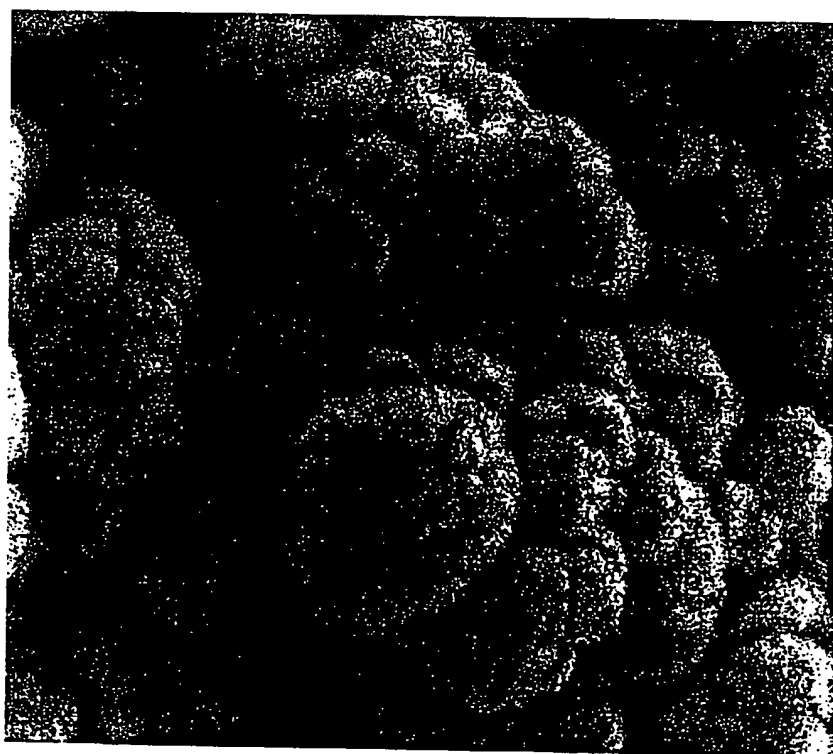
500 nm

**FIG. 7**



500 nm

*FIG. 8*



500 nm

**FIG. 9**

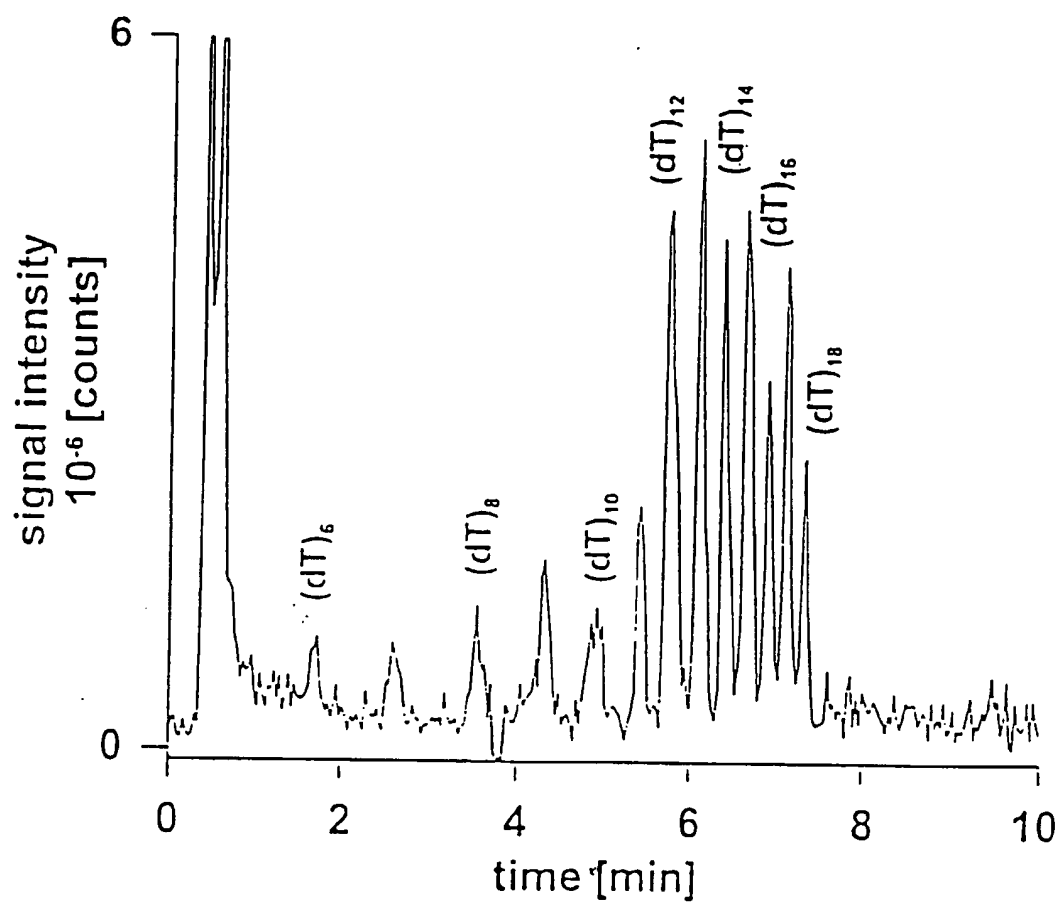


FIG. 10

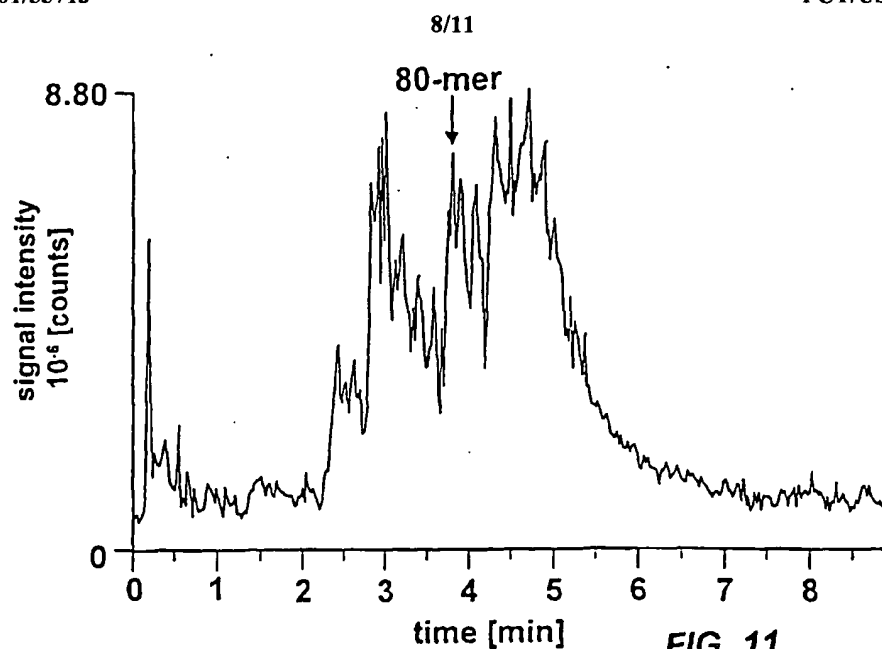


FIG. 11

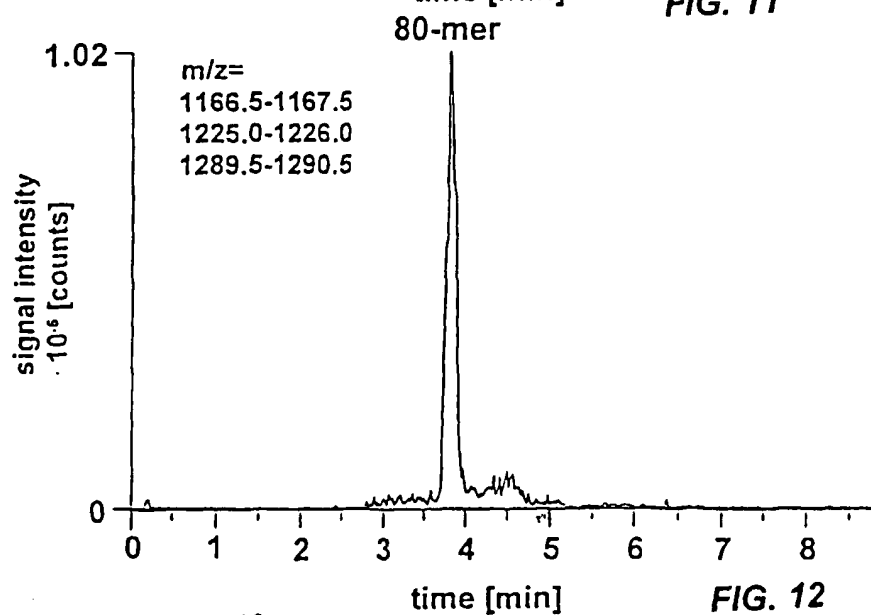


FIG. 12

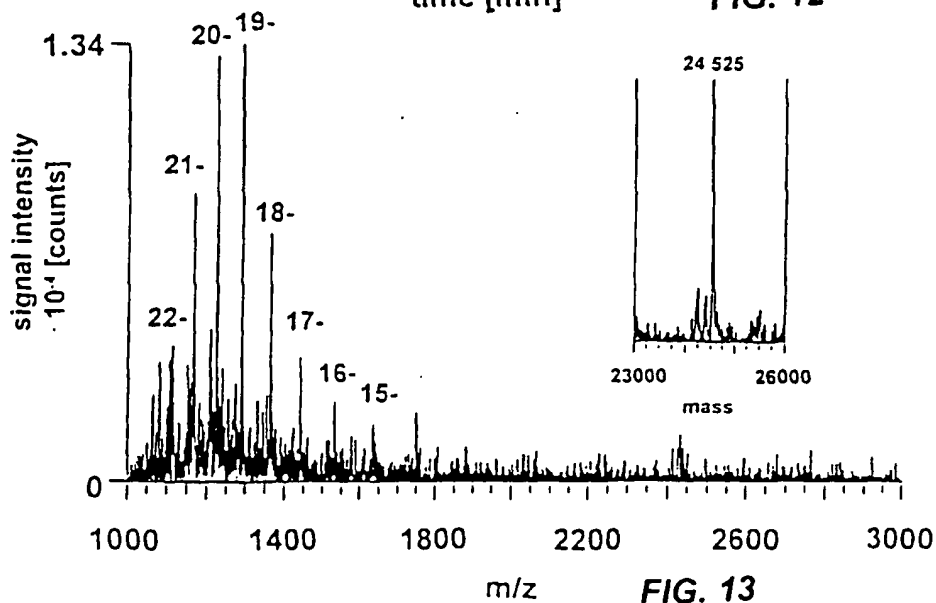


FIG. 13

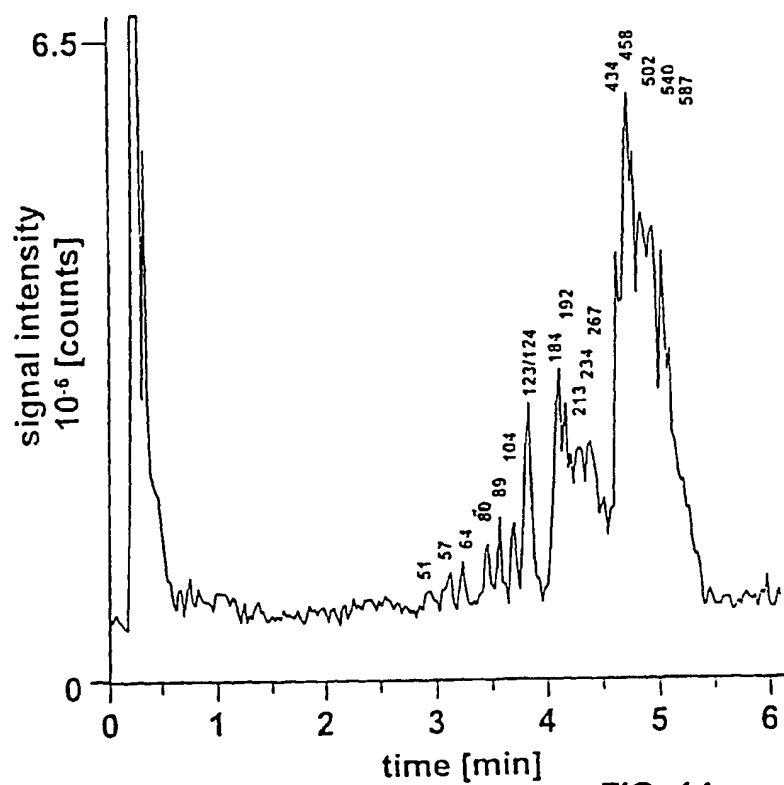
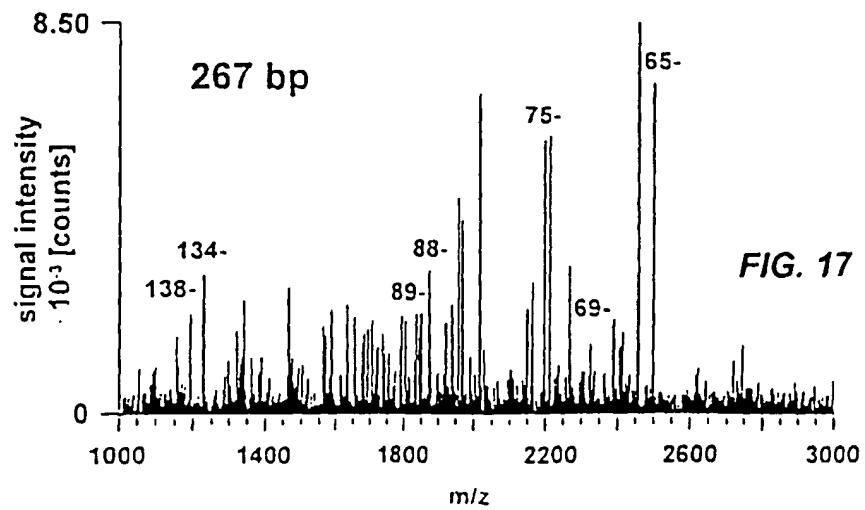
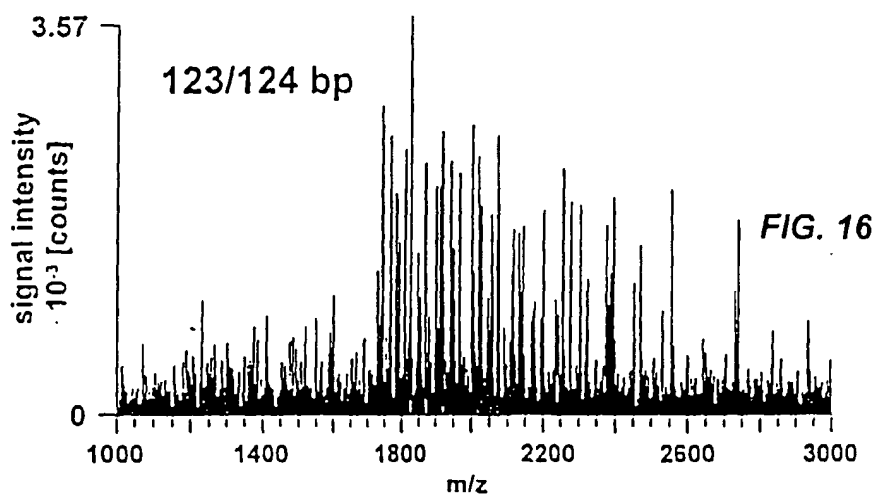
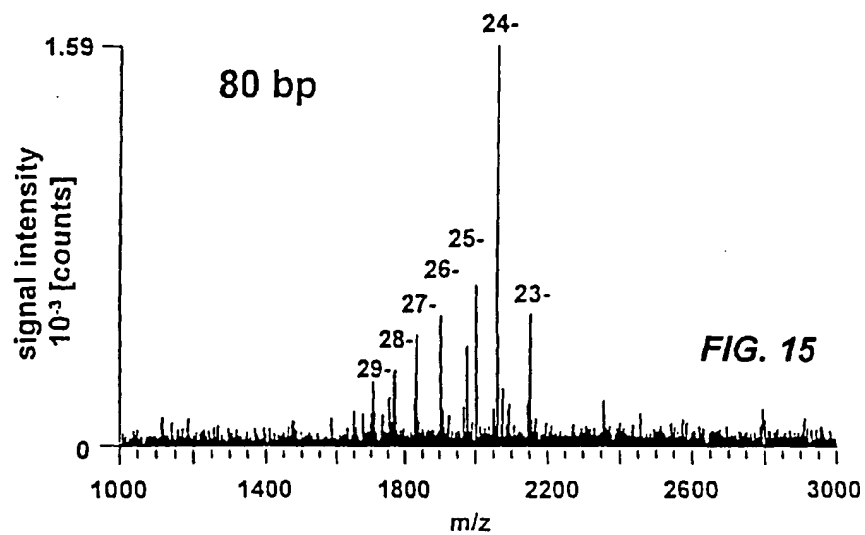


FIG. 14



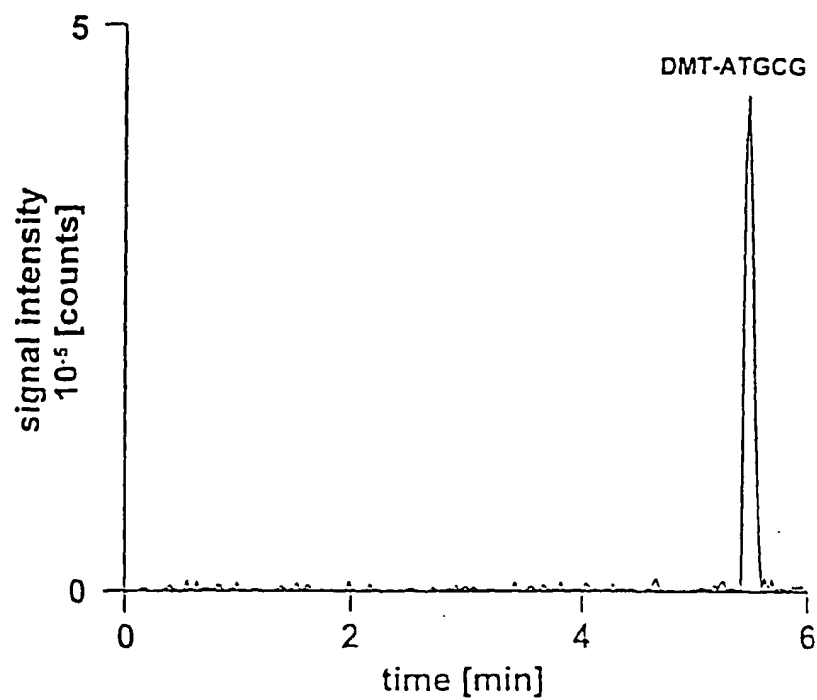


FIG. 18

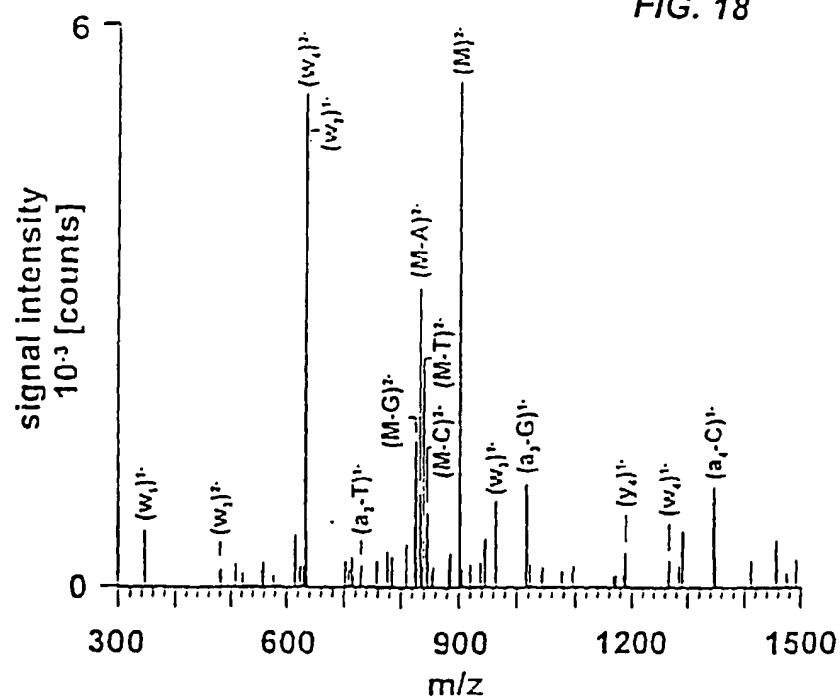


FIG. 19

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
2 August 2001 (02.08.2001)

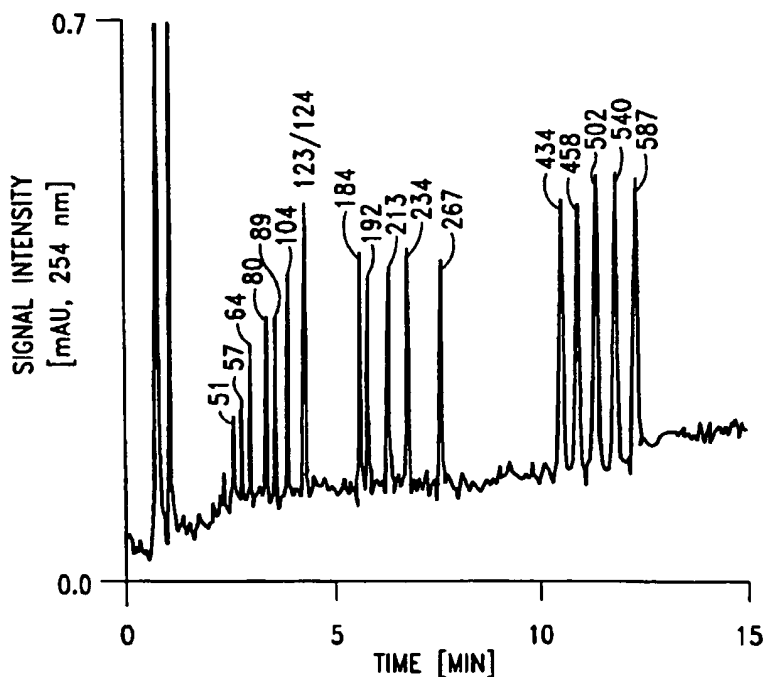
PCT

(10) International Publication Number  
**WO 01/55713 A3**

- (51) International Patent Classification<sup>7</sup>: C07K 1/36. B01D 15/08, B01J 20/26
- (21) International Application Number: PCT/US01/02539
- (22) International Filing Date: 25 January 2001 (25.01.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/178,553 26 January 2000 (26.01.2000) US
- (71) Applicant: TRANSGENOMIC, INC. [US/US]; 2032 Concourse Drive, San Jose, CA 95131 (US).
- (72) Inventors: HUBER, Christian; Ulmanstrasse 65, A-6063 Rum (AT). OBERARCHER, Herbert; Schulstrasse 17, A-6161 Natters (AT). PREMSTALLER, Andreas; Andreas-Hofer Strasse 2, I-39012 Meran (IT).
- (74) Agents: WALKER, William, B. et al.; Transgenomic, Inc., 2032 Concourse Drive, San Jose, CA 95131 (US).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:  
— with international search report

[Continued on next page]

(54) Title: METHOD AND APPARATUS FOR SEPARATING POLYNUCLEOTIDES USING MONOLITHIC CAPILLARY COLUMNS



(57) Abstract: Methods and devices based on capillary monolithic columns, preferably consisting of an underivatized poly(styrene-divinylbenzene) monolith, for separating a mixture of polynucleotides by ion pair-reverse phase-high performance chromatography (IP-RP-HPLC). In various aspects of the method and device the monolith is characterized by one or more of the following: the monolith is contained within a capillary tube; the monolith is immobilized by covalent attachment at the inner wall of the tube; the tube is devoid of retaining frits; the monolith is characterized by having above 10,000 theoretical plates per meter and preferably above 200,000 theoretical plates per meter; the method uses a mobile phase which is devoid of EDTA; the monolith has a surface morphology that is rugulose or brush-like; the chromatographic surfaces of the monolith are non-porous; the monolith has channels sufficiently

large for convective flow of the mobile phase; the monolith is formed from a polymerization mixture including underivatized styrene, a crosslinking agent, and a porogen, wherein the porogen includes tetrahydrofuran. The monolith can be incorporated into a miniaturized chromatography system which can be coupled to a mass spectrometer for on-line separation and mass determination of single- or double-stranded polynucleotides.

WO 01/55713 A3



(88) Date of publication of the international search report:  
14 March 2002

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# INTERNATIONAL SEARCH REPORT

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                                                                                                                                                 |                                                                                                         |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|
| International Application No<br><b>PCT/US 01/02539</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                                                                                                                                                 |                                                                                                         |
| <b>A. CLASSIFICATION OF SUBJECT MATTER</b><br>IPC 7 C07K1/36 B01D15/08 B01J20/26                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                 |                                                                                                         |
| According to International Patent Classification (IPC) or to both national classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                 |                                                                                                         |
| <b>B. FIELDS SEARCHED</b><br>Minimum documentation searched (classification system followed by classification symbols)<br>IPC 7 C07K G01N B01J                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                                                                                                                                                                                                                                                 |                                                                                                         |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                 |                                                                                                         |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used)<br>EPO-Internal                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                                                                                 |                                                                                                         |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                 |                                                                                                         |
| Category *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                              | Relevant to claim No.                                                                                   |
| A                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | US 5 972 222 A (TOGAMI DAVID W ET AL)<br>26 October 1999 (1999-10-26)<br><br>column 1, line 12-182<br>column 2, line 24-45<br>column 5, line 66 -column 6, line 13<br>column 15, line 67<br><div style="text-align: center;">---<br/>-/--</div> | 1,9,16,<br>23,30,<br>37,44,<br>51,59,<br>67,73,<br>79,86,<br>96,97                                      |
| <div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.         </div> <div> <input checked="" type="checkbox"/> Patent family members are listed in annex.         </div> </div>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                 |                                                                                                         |
| * Special categories of cited documents :<br><div style="display: flex;"> <div style="flex: 1;"> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>*Z* document member of the same patent family</p> </div> </div> |                                                                                                                                                                                                                                                 |                                                                                                         |
| Date of the actual completion of the international search<br><div style="text-align: center;">9 November 2001</div>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                 | Date of mailing of the international search report<br><div style="text-align: center;">20/11/2001</div> |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.<br>Fax: (+31-70) 340-3016                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                                                                                                                                                 | Authorized officer<br><div style="text-align: center;">Zinngrebe, U</div>                               |

## PC r/US 01/02539

page 2 of 3

## INTERNATIONAL SEARCH REPORT

In' tional Application No

PCT/US 01/02539

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inventor's International Application No

PCT/US 01/02539

| Patent document<br>cited in search report |   | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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|                                           |   |                     | WO 9856798 A1              | 17-12-1998          |
| -----                                     |   |                     |                            |                     |
| US 5728457                                | A | 17-03-1998          | NONE                       |                     |
| -----                                     |   |                     |                            |                     |

CORRECTED VERSION

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
2 August 2001 (02.08.2001)

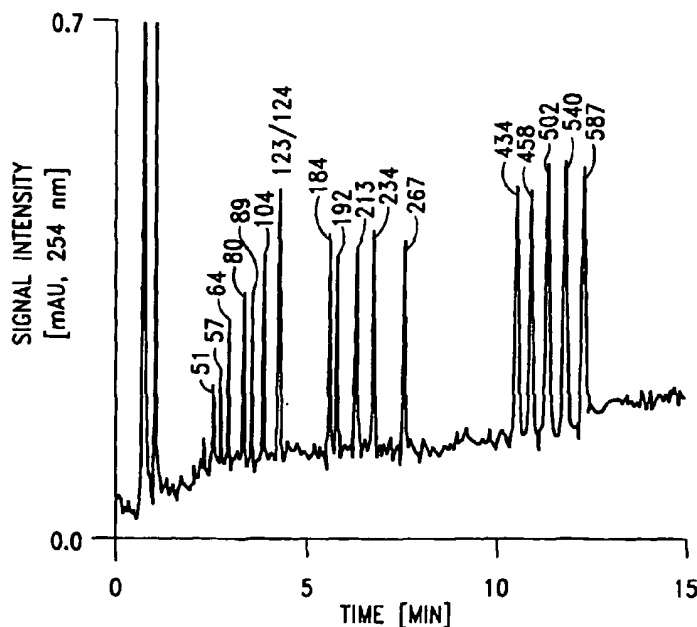
PCT

(10) International Publication Number  
**WO 01/055713 A3**

- (51) International Patent Classification<sup>7</sup>: C07K 1/36, B01D 15/08, B01J 20/26 (72) Inventors: HUBER, Christian; Ulmanestrasse 65, A-6063 Rum (AT). OBERARCHER, Herbert; Schulstrasse 17, A-6161 Natters (AT). PREMSTALLER, Andreas; Andreas-Hofer Strasse 2, I-39012 Meran (IT).
- (21) International Application Number: PCT/US01/02539
- (22) International Filing Date: 25 January 2001 (25.01.2001) (74) Agents: WALKER, William, B. et al.; Transgenomic, Inc., 2032 Concourse Drive, San Jose, CA 95131 (US).
- (25) Filing Language: English (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (26) Publication Language: English
- (30) Priority Data: 60/178,553 26 January 2000 (26.01.2000) US (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
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[Continued on next page]

(54) Title: METHOD AND APPARATUS FOR SEPARATING POLYNUCLEOTIDES USING MONOLITHIC CAPILLARY COLUMNS



(57) Abstract: Methods and devices based on capillary monolithic columns, preferably consisting of an underivatized poly(styrene-divinylbenzene) monolith, for separating a mixture of polynucleotides by ion pair-reverse phase-high performance chromatography (IP-RP-HPLC). In various aspects of the method and device the monolith is characterized by one or more of the following: the monolith is contained within a capillary tube; the monolith is immobilized by covalent attachment at the inner wall of the tube; the tube is devoid of retaining frits; the monolith is characterized by having above 10,000 theoretical plates per meter and preferably above 200,000 theoretical plates per meter; the method uses a mobile phase which is devoid of EDTA; the monolith has a surface morphology that is rugulose or brush-like; the chromatographic surfaces of the monolith are non-porous; the

monolith has channels sufficiently large for convective flow of the mobile phase; the monolith is formed from a polymerization mixture including underivatized styrene, a crosslinking agent, and a porogen, wherein the porogen includes tetrahydrofuran. The monolith can be incorporated into a miniaturized chromatography system which can be coupled to a mass spectrometer for on-line separation and mass determination of single- or double-stranded polynucleotides.

WO 01/055713 A3



patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**(48) Date of publication of this corrected version:**

31 October 2002

**(15) Information about Correction:**

see PCT Gazette No. 44/2002 of 31 October 2002, Section II

**Published:**

— with international search report

**(88) Date of publication of the international search report:**

14 March 2002

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**TITLE OF THE INVENTION**

**METHOD AND APPARATUS FOR SEPARATING POLYNUCLEOTIDES USING  
MONOLITHIC CAPILLARY COLUMNS**

**FIELD OF THE INVENTION**

The present invention relates to methods and devices for analyzing polynucleotides. In particular, the invention relates to the use of monolithic capillary columns for use in high-performance liquid chromatography of single and double-stranded polynucleotides.

**BACKGROUND OF THE INVENTION**

Genetics and proteomics depend on the ability to analyze complex mixtures of biological origin with high sensitivity and maximum selectivity. Especially the rapid development of miniaturized techniques in analytical chemistry (He et al. *Anal. Chem.* 70:3790-3797 (1998)) has had a profound impact on the modern practice of analyzing biological samples of high complexity (Novotny *J. Chromatogr. B* 689: 55-70 (1997)). Several techniques based on the principle of differential migration (Rathore et al. *J. Chromatogr. A* 743: 231-246 (1996)) were developed after the introduction of fused silica capillaries to analytical chemistry (Dandeneau et al. *HRC & CC*: 2:351 (1979)), in particular capillary liquid chromatography (CLC) (Hirata et al. *J. Chromatogr.* 186:521-528 (1979)), capillary electrophoresis (CE) (Jorgenson et al. *J. Chromatogr.* 218:209-216 (1981)), and capillary electrochromatography (CEC) (Jorgenson et al. *J. Chromatogr.* 218:209-216 (1981)).

Columns packed with microparticulate sorbents have been successfully applied as separation media in high-performance liquid chromatography (HPLC). Despite many advantages, HPLC columns packed with microparticulate, porous stationary phases have some limitations, such as the relatively large void volume between the packed particles and the slow diffusional mass transfer of solutes into and out of the stagnant mobile phase present in the pores of the separation medium (Martin et al. *Biochem J.* 35:1358 (1941); Unger et al in *Packings and Stationary Phases in Chromatographic Techniques*, Unger Ed: Marcel Dekker: New York, p. 75 (1990)).

One approach to alleviate the problem of restricted mass transfer and intraparticle void volume is the concept of monolithic chromatographic beds, where the separation medium consists of a continuous rod of a rigid, polymer which has no interstitial volume but only internal porosity consisting of micropores and macropores. Monolithic separation columns are becoming more widely used in HPLC of biomolecules.

1 WO 97/19347 relates to a method and device for separating one or several organic  
2 substances in a sample. The chromatographic device comprises a monolith prepared in  
3 an emulsion system containing at least 75% by weight of water phase. Separations of  
4 polynucleotides were not disclosed.

5 U.S. 5,334,310 relates to a monolith containing small pores having diameters less  
6 than about 200 nm and large pores with diameters greater than about 600 nm. The  
7 columns were equipped with end fittings. No separations of polynucleotides were  
8 demonstrated.

9 WO 00/15778 relates to polymeric monolithic beds for resolving mixtures containing  
10 polynucleotides. However, single-stranded molecules were poorly resolved using the  
11 column. The columns had inner diameters (ID) of greater than 4mm and were equipped  
12 retaining frits. The mobile phase buffers included EDTA. Useful separations of DNA  
13 fragments by IP-RP-HPLC using underivatized polystyrene/divinylbenzene monolithic  
14 columns could not be achieved and such columns were not recommended.

15 There is a need for improved monolithic columns and methods for the separation  
16 of polynucleotides.

## 17 SUMMARY OF THE INVENTION

18 In one aspect, the present invention provides a method for separating a mixture  
19 of polynucleotides. The method includes applying the mixture of polynucleotides to a  
20 polymeric monolith having non-polar chromatographic surfaces and eluting the mixture  
21 of polynucleotides with a mobile phase including a counterion agent and an organic  
22 solvent, wherein the monolith is an underivatized poly(styrene-divinylbenzene) matrix.  
23 In the method, the monolith preferably is contained within a fused silica tube having an  
24 inner diameter in the range of 1 to 1000 micrometer and the monolith is immobilized by  
25 covalent attachment at the inner wall of the tube. The tube is preferably devoid of  
26 retaining frits. In preferred embodiments of this aspect of the invention, the monolith is  
27 characterized by having 100,000 to 200,000 theoretical plates per meter. The  
28 theoretical plates per meter can be determined from the retention time of single stranded  
29 p(dT)<sub>18</sub> standard using the following equation:

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

30 wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
31 standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half height, and  
32  $L$  is the length of the monolith in meters. In one embodiment, during the isocratic

1 elution, the back pressure was about 180 to 200 bar, at a flow rate in the range of 2 to 3  
2  $\mu\text{L}/\text{min}$  and at an elution temperature of  $50^{\circ}\text{C}$  for a monolith having an ID of 200  
3 micrometer and a length of 60 mm. The method can be performed using a mobile  
4 phase which is devoid of EDTA. The preferred monolith has a surface morphology, as  
5 determined by scanning electron microscopy, that resembles the surface morphology of  
6 octadecyl modified poly(styrene-divinylbenzene) particles, wherein the surface  
7 morphology of the monolith is rugulose. Additionally, the preferred monolith has a  
8 surface morphology, as determined by scanning electron microscopy, that resembles  
9 the surface morphology of octadecyl modified poly(styrene-divinylbenzene) particles,  
10 wherein the surface morphology of the monolith is brush-like. The monolith can be  
11 formed from a polymerization mixture including underivatized styrene, a crosslinking  
12 agent, and a porogen, wherein the porogen includes tetrahydrofuran. A preferred  
13 porogen includes a mixture of tetrahydrofuran and decanol. In the method, the  
14 polynucleotides can include double-stranded fragments having lengths in the range of 3  
15 to 600 base pairs. The method can further include analyzing eluted polynucleotides by  
16 mass spectral analysis. In the method, the monolith preferably has a back pressure in  
17 the range of about 20 to about 300 bar, and typically in the range of about 70 to about  
18 200 bar. The method can be performed at a monolith temperature in the range of about  
19  $20^{\circ}\text{C}$  to about  $90^{\circ}\text{C}$ .

20 In another aspect, the invention concerns a method for separating a mixture of  
21 polynucleotides. The method includes applying the mixture of polynucleotides to a  
22 polymeric monolith having non-polar chromatographic surfaces and eluting the mixture  
23 of polynucleotides with a mobile phase comprising a counterion agent and an organic  
24 solvent. In a preferred embodiment, the monolith comprises an underivatized  
25 poly(styrene-divinylbenzene) matrix. In this aspect of the invention, the monolith is  
26 preferably contained within a fused silica tube, and the monolith is immobilized by  
27 covalent attachment at the inner wall of the tube. The tube can have an inner diameter  
28 in the range of 10 micrometer to 1000 micrometer, and preferably in the range of 1  
29 micrometer to 1000 micrometer. The tube is preferably devoid of retaining frits. In  
30 certain embodiments, the monolith is characterized by having 10,000 to 200,000  
31 theoretical plates per meter and preferably characterized by having 100,000 to 200,000  
32 theoretical plates per meter. During the elution, the mobile phase preferably is devoid of  
33 EDTA. The preferred monolith has a surface morphology, as determined by scanning  
34 electron microscopy, that resembles the surface morphology of octadecyl modified  
35 poly(styrene-divinylbenzene) particles, wherein the surface morphology of the monolith

1 is rugulose. The monolith can be formed from a polymerization mixture including  
2 underivatized styrene, a crosslinking agent, and a porogen, wherein the porogen  
3 comprises tetrahydrofuran.

4 In another aspect, the invention provides a method for separating a mixture of  
5 polynucleotides. The method includes applying the mixture of polynucleotides to a  
6 polymeric monolith having non-polar chromatographic surfaces and eluting the mixture  
7 of polynucleotides with a mobile phase comprising a counterion agent and an organic  
8 solvent, wherein the monolith comprises an underivatized poly(styrene-divinylbenzene)  
9 matrix, wherein the monolith is contained within a fused silica tube, and wherein the  
10 tube is devoid of retaining frits, wherein the tube has an inner diameter in the range of 1  
11 micrometer to 1000 micrometer, and wherein the polynucleotides are double-stranded  
12 fragments having lengths in the range of 3 to 600 base pairs. During the elution, the  
13 mobile phase preferably is devoid of EDTA. The monolith preferably is immobilized by  
14 covalent attachment at the inner wall of the tube. In certain embodiments, the monolith  
15 is characterized by having 50,000 to 200,000 theoretical plates per meter. In preferred  
16 embodiments, the monolith is characterized by having greater than about 190,000  
17 theoretical plates per meter. The preferred monolith has a surface morphology, as  
18 determined by scanning electron microscopy, that resembles the surface morphology of  
19 octadecyl modified poly(styrene-divinylbenzene) particles, wherein the surface  
20 morphology of the monolith is rugulose.

21 In a further aspect, the invention provides a method for separating a mixture of  
22 polynucleotides. The method includes applying the mixture of polynucleotides to a  
23 polymeric monolith having non-polar chromatographic surfaces and eluting said mixture  
24 of polynucleotides with a mobile phase comprising a counterion agent and an organic  
25 solvent, wherein the monolith is characterized by having 10,000 to 200,000 theoretical  
26 plates per meter, wherein the monolith includes an underivatized poly(styrene-  
27 divinylbenzene) matrix, wherein the monolith is contained within a fused silica tube  
28 having an inner diameter in the range of 1 micrometer to 1000 micrometer, and wherein  
29 the monolith is immobilized by covalent attachment at the inner wall of the tube. In a  
30 preferred embodiment, the theoretical plates per meter is determined from the retention  
31 time of single stranded p(dT)<sub>18</sub> standard using the following equation:

$$(N / L) = (5.54 / L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

1        wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
2        standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half height, and  
3         $L$  is the length of the monolith in meters. The tube preferably is devoid of retaining frits.  
4        In the method, the mobile phase preferably is devoid of EDTA. In a preferred  
5        embodiment, the monolith has a surface morphology, as determined by scanning  
6        electron microscopy, that resembles the surface morphology of octadecyl modified  
7        poly(styrene-divinylbenzene) particles, wherein the surface morphology of said monolith  
8        is rugulose. Also in a preferred embodiment, the monolith has a surface morphology, as  
9        determined by scanning electron microscopy, that resembles the surface morphology of  
10       octadecyl modified poly(styrene-divinylbenzene) particles, wherein the surface  
11       morphology of said monolith is brush-like.

12       In a yet further aspect, the invention concerns a method for separating a mixture  
13       of polynucleotides. The method includes applying the mixture of polynucleotides to a  
14       polymeric monolith having non-polar chromatographic surfaces and eluting the mixture  
15       of polynucleotides with a mobile phase comprising a counterion agent and an organic  
16       solvent, and wherein the mobile phase is devoid of EDTA. In this aspect, the monolith  
17       preferably is contained within a fused silica tube having an inner diameter in the range  
18       of 10 micrometer to 1000 micrometer. The monolith preferably is immobilized by  
19       covalent attachment at the inner wall of the tube. The tube preferably is devoid of  
20       retaining frits. In certain embodiments of this aspect of the invention, the monolith is  
21       characterized by having 10,000 to 200,000 theoretical plates per meter. The preferred  
22       monolith has a surface morphology, as determined by scanning electron microscopy,  
23       that resembles the surface morphology of octadecyl modified poly(styrene-  
24       divinylbenzene) particles, wherein the surface morphology of the monolith is rugulose.  
25       The preferred monolith comprises an underivatized poly(styrene-divinylbenzene) matrix.

26       In a still further aspect, the invention provides a method for separating a mixture  
27       of polynucleotides. The method includes applying the mixture of polynucleotides to a  
28       polymeric monolith having non-polar chromatographic surfaces and eluting the mixture  
29       of polynucleotides with a mobile phase comprising a counterion agent and an organic  
30       solvent, wherein the monolith has a surface morphology, as determined by scanning  
31       electron microscopy, that resembles the surface morphology of octadecyl modified  
32       poly(styrene-divinylbenzene) particles, wherein the surface morphology of the monolith  
33       is rugulose, and wherein the monolith comprises an underivatized poly(styrene-  
34       divinylbenzene) matrix. The mobile phase preferably is devoid of EDTA. In preferred  
35       embodiments, the monolith can be characterized by one or more of the following: the

1 monolith is contained within a fused silica tube having an inner diameter in the range of  
2 1 micrometer to 1000 micrometer; the monolith is immobilized by covalent attachment at  
3 the inner wall of the tube; and, the tube is devoid of retaining frits. In preferred  
4 embodiments, the monolith is characterized by having 100,000 to 200,000 theoretical  
5 plates per meter.

6 In a related aspect, the invention provides a method for separating a mixture of  
7 polynucleotides. In this aspect, the method includes applying the mixture of  
8 polynucleotides to a polymeric monolith having non-polar chromatographic surfaces and  
9 eluting the mixture of polynucleotides with a mobile phase comprising a counterion  
10 agent and an organic solvent, wherein the monolith comprises an underivatized  
11 poly(styrene-divinylbenzene) matrix, wherein the monolith is contained within a fused  
12 silica tube having an inner diameter in the range of 1 micrometer to 1000 micrometer,  
13 wherein the monolith is immobilized at the inner wall of the tube, and wherein the tube is  
14 devoid of retaining frits. Preferred embodiments of this aspect of the invention can  
15 include one or more of the following: the mobile phase is devoid of EDTA; the monolith  
16 is characterized by having 100,000 to 200,000 theoretical plates per meter; and, the  
17 monolith has a surface morphology, as determined by scanning electron microscopy,  
18 that resembles the surface morphology of octadecyl modified poly(styrene-  
19 divinylbenzene) particles, wherein the surface morphology of the monolith is rugulose.  
20 The monolith can be formed from a polymerization mixture including underivatized  
21 styrene, a crosslinking agent, and a porogen, wherein the porogen comprises  
22 tetrahydrofuran. The method can further include analyzing eluted polynucleotides by  
23 mass spectral analysis.

24 In an additional aspect, the invention provides a device for separating a mixture  
25 of polynucleotides. The device includes a polymeric monolith having non-polar  
26 chromatographic surfaces, wherein the monolith is contained within a fused silica tube  
27 having an inner diameter in the range of 1 micrometer to 1000 micrometer, wherein the  
28 monolith is immobilized by covalent attachment at the inner wall of the tube, and  
29 wherein the monolith comprises an underivatized poly(styrene-divinylbenzene) matrix.  
30 Preferred embodiments of this aspect of the invention can be further characterized by  
31 the following: the tube is devoid of retaining frits; the monolith is characterized by having  
32 100,000 to 200,000 theoretical plates per meter. The theoretical plates per meter  
33 preferably is determined from the retention time of single stranded p(dT)<sub>18</sub> standard  
34 using the following equation:

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

1            wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
2            standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half height, and  
3             $L$  is the length of the monolith in meters. During the isocratic elution the monolith  
4            preferably has a back pressure of 180 to 200 bar, and a flow rate in the range of 2 to 3  
5             $\mu\text{L}/\text{min}$  at an elution temperature of  $50^\circ\text{C}$ . Preferred embodiments of the device can be  
6            characterized by one or more of the following: the chromatographic surfaces of the  
7            monolith are non-porous; the monolith has channels sufficiently large for convective flow  
8            of said mobile phase; and, the monolith can be formed from a polymerization mixture  
9            including underivatized styrene, a crosslinking agent, and a porogen, wherein the  
10           porogen comprises tetrahydrofuran.

11           In a further yet aspect, the invention concerns a device for separating a mixture  
12           of polynucleotides. The device includes a polymeric monolith having non-polar  
13           chromatographic surfaces, wherein the monolith is contained within a fused silica tube,  
14           wherein the monolith is immobilized by covalent attachment at the inner wall of the tube,  
15           and wherein the monolith comprises an underivatized poly(styrene-divinylbenzene)  
16           matrix. Preferred embodiments can include one or more of the following features: the  
17           tube has an inner diameter in the range of 1 micrometer to 1000 micrometer; the tube is  
18           devoid of retaining frits; the monolith is characterized by having 10,000 to 200,000  
19           theoretical plates per meter; the monolith comprises an underivatized monolithic  
20           stationary phase; the monolith has a surface morphology, as determined by scanning  
21           electron microscopy, that resembles the surface morphology of octadecyl modified  
22           poly(styrene-divinylbenzene) particles, wherein the surface morphology of the monolith  
23           is rugulose; the chromatographic surfaces of the monolith are non-porous; and the  
24           particles have channels sufficiently large for convective flow of the mobile phase. The  
25           monolith can be formed from a polymerization mixture including underivatized styrene, a  
26           crosslinking agent, and a porogen, wherein the porogen comprises tetrahydrofuran.

27           In another aspect, the invention concerns a device for separating a mixture of  
28           polynucleotides. The device includes a polymeric monolith having non-polar  
29           chromatographic surfaces, wherein the monolith is contained within a fused silica tube,  
30           wherein the tube is devoid of retaining frits, and wherein the monolith comprises an  
31           underivatized poly(styrene-divinylbenzene) matrix. Preferred embodiments of this  
32           aspect of the invention can further include one or more of the following: the monolith is

1 immobilized by covalent attachment at the inner wall of said tube; the monolith is  
2 characterized by having 100,000 to 200,000 theoretical plates per meter; the tube has  
3 an inner diameter in the range of 1 micrometer to 1000 micrometer; and, the monolith  
4 has a surface morphology, as determined by scanning electron microscopy, that  
5 resembles the surface morphology of octadecyl modified poly(styrene-divinylbenzene)  
6 particles, wherein the surface morphology of the monolith is rugulose. The monolith can  
7 be formed from a polymerization mixture including underivatized styrene, a crosslinking  
8 agent, and a porogen, wherein the porogen comprises tetrahydrofuran.

9 In a related aspect, the invention provides a device for separating a mixture of  
10 polynucleotides. The device includes a polymeric monolith having non-polar  
11 chromatographic surfaces, wherein the monolith is characterized by having 100,000 to  
12 200,000 theoretical plates per meter, wherein the monolith is contained within a fused  
13 silica tube having an inner diameter in the range of 1 micrometer to 1000 micrometer,  
14 and wherein the tube has been silianized. Preferred embodiments of this aspect of the  
15 invention can further include one or more of the following: the monolith is immobilized  
16 by covalent attachment at the inner wall of the tube; the tube is devoid of retaining frits;  
17 the monolith comprises an underivatized poly(styrene-divinylbenzene) matrix; and, the  
18 monolith has a surface morphology, as determined by scanning electron microscopy,  
19 that resembles the surface morphology of octadecyl modified poly(styrene-  
20 divinylbenzene) particles, wherein the surface morphology of the monolith is rugulose.  
21 The monolith can be formed from a polymerization mixture including underivatized  
22 styrene, a crosslinking agent, and a porogen, wherein the porogen comprises  
23 tetrahydrofuran.

24 In an important aspect, the invention provides a device for separating a mixture  
25 of polynucleotides. The device includes a polymeric monolith having non-polar  
26 chromatographic surfaces, wherein the monolith comprises an underivatized  
27 poly(styrene-divinylbenzene) matrix, and wherein the monolith is characterized by  
28 having 10,000 to 200,000 theoretical plates per meter. Preferred embodiments of this  
29 aspect of the invention can further include one or more of the following: the monolith is  
30 contained within a tube having an inner diameter in the range of 1 micrometer to 1000  
31 micrometer; the monolith is immobilized at the inner wall of the tube; the tube is devoid  
32 of retaining frits; and, the monolith has a surface morphology, as determined by  
33 scanning electron microscopy, that resembles the surface morphology of octadecyl  
34 modified poly(styrene-divinylbenzene) particles, wherein the surface morphology of the  
35 monolith is rugulose.

1 In another aspect, the invention provides a miniaturized chromatographic system  
2 for separating a mixture of polynucleotides. The device includes a polymeric monolith  
3 having non-polar chromatographic surfaces, wherein the monolith comprises an  
4 underivatized poly(styrene-divinylbenzene) matrix, wherein the monolith is  
5 characterized by having at least 100,000 theoretical plates per meter, wherein the  
6 monolith is contained within a tube having an inner diameter in the range of 10  
7 micrometer to 1000 micrometer, and wherein the monolith is immobilized at the inner  
8 wall of the tube. Preferred embodiments of this aspect of the invention can further  
9 include one or more of the following: the tube is devoid of retaining frits; the monolith is  
10 contained within a tube having an inner diameter in the range of 1 micrometer to 1000  
11 micrometer; the monolith has a surface morphology, as determined by scanning  
12 electron microscopy, that resembles the surface morphology of octadecyl modified  
13 poly(styrene-divinylbenzene) particles, wherein the surface morphology of the monolith  
14 is rugulose; and wherein the monolith has a surface morphology, as determined by  
15 scanning electron microscopy, that resembles the surface morphology of octadecyl  
16 modified poly(styrene-divinylbenzene) particles, wherein the surface morphology of the  
17 monolith is brush-like. The monolith can be formed from a polymerization mixture  
18 including underivatized styrene, a crosslinking agent, and a porogen, wherein the  
19 porogen comprises tetrahydrofuran.

20 In an additional aspect, the invention concerns a miniaturized chromatographic  
21 system for separating a mixture of polynucleotides. The system preferably includes a  
22 device which includes a polymeric monolith having non-polar chromatographic surfaces,  
23 wherein the monolith comprises an underivatized poly(styrene-divinylbenzene) matrix,  
24 wherein the monolith is characterized by having at least 100,000 theoretical plates per  
25 meter, wherein the monolith is contained within a tube having an inner diameter in the  
26 range of 10 micrometer to 1000 micrometer, and wherein the monolith is immobilized at  
27 the inner wall of the tube. In the system, the monolith can be operatively coupled to a  
28 mass spectrometer.

29

30 In a further aspect, the invention concerns a device for separating a mixture of  
31 polynucleotides. The device includes a polymeric monolith having non-polar  
32 chromatographic surfaces, wherein the monolith has a surface morphology, as  
33 determined by scanning electron microscopy, that resembles the surface morphology of  
34 octadecyl modified poly(styrene-divinylbenzene) particles, wherein the surface  
35 morphology of the monolith is rugulose and brush-like, wherein the monolith is

1 contained within a fused silica tube having an inner diameter in the range of 1  
2 micrometer to 1000 micrometer, and wherein the monolith is immobilized at the inner  
3 wall of said tube. Preferred embodiments of this aspect of the invention can further  
4 include one or more of the following: the tube is devoid of retaining frits; the monolith is  
5 characterized by having 100,000 to 200,000 theoretical plates per meter; the monolith  
6 comprises an underivatized poly(styrene-divinylbenzene) matrix; and, the surface of  
7 said monolith is non-porous. The monolith can be formed from a polymerization mixture  
8 including underivatized styrene, a crosslinking agent, and a porogen, wherein the  
9 porogen comprises tetrahydrofuran. The polynucleotides can include double-stranded  
10 fragments having lengths in the range of 3 to 2000 base pairs, and preferably 3 to 600  
11 base pairs.

12 In a final aspect, the invention concerns a chromatographic device. The device  
13 includes a polymeric monolith having non-polar chromatographic surfaces, wherein the  
14 monolith comprises an underivatized poly(styrene-divinylbenzene) matrix, wherein the  
15 monolith is characterized by having at least 10,000 theoretical plates per meter, wherein  
16 the monolith is contained within a silanized fused silica tube having an inner diameter in  
17 the range of 10 micrometer to 1000 micrometer, and wherein the monolith is  
18 immobilized at the inner wall of the tube. Preferred embodiments of this aspect of the  
19 invention can be further characterized by the following: the tube is devoid of retaining  
20 frits; the monolith is characterized by having 100,000 to 200,000 theoretical plates per  
21 meter; the monolith has a surface morphology, as determined by scanning electron  
22 microscopy, that resembles the surface morphology of octadecyl modified poly(styrene-  
23 divinylbenzene) particles, wherein the surface morphology of the monolith is rugulose;  
24 and wherein the monolith has a surface morphology, as determined by scanning  
25 electron microscopy, that resembles the surface morphology of octadecyl modified  
26 poly(styrene-divinylbenzene) particles, wherein the surface morphology of the monolith  
27 is brush-like. The monolith can be formed from a polymerization mixture including  
28 underivatized styrene, a crosslinking agent, and a porogen, wherein the porogen  
29 comprises tetrahydrofuran. The theoretical plates per meter preferably is determined  
30 from the retention time of single stranded p(dT)<sub>18</sub> standard using the following equation:

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

31 wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
32 standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half height, and

1  $L$  is the length of the monolith in meters. During the isocratic elution the monolith  
2 preferably has a back pressure of 180 to 200 bar, and a flow rate in the range of 2 to 3  
3  $\mu\text{L}/\text{min}$  at an elution temperature of  $50^{\circ}\text{C}$ . Preferred embodiments of the device can be  
4 characterized by one or more of the following: the chromatographic surfaces of the  
5 monolith are non-porous; the monolith has channels sufficiently large for convective flow  
6 of said mobile phase; and, the monolith can be formed from a polymerization mixture  
7 including underivatized styrene, a crosslinking agent, and a porogen, wherein the  
8 porogen comprises tetrahydrofuran. The device can be used with back pressures in the  
9 range of about 20 to 300 bar, and with temperatures in the range of  $20^{\circ}\text{C}$  to about  $90^{\circ}\text{C}$ .

#### 10 BRIEF DESCRIPTION OF THE DRAWINGS

11 FIG. 1 illustrates a method for derivatization of a capillary silica wall by (a) vinyl-  
12 silanization and (b) subsequent grafting of the forming polymer.

13 FIG. 2 is a chromatogram showing capillary ion-pair reverse phase-high  
14 pressured liquid chromatography (IP-RP-HPLC) separation of phosphorylated  
15 polynucleotide ladders (0.66 - 1.64 fmol of each polynucleotide) in a monolithic capillary  
16 column constructed in accordance with an embodiment of the present invention.

17 FIG. 3 is a chromatogram showing capillary ion-pair reverse phase-high  
18 pressured liquid chromatography (IP-RP-HPLC) separation of phosphorylated  
19 polynucleotide ladders (40 - 98 fmol of each polynucleotide) in a monolithic capillary  
20 column constructed in accordance with an embodiment of the present invention.

21 FIG. 4 is a chromatogram showing capillary IP-RP-HPLC separation of  
22 phosphorylated and dephosphorylated deoxyadenylic acids in a monolithic capillary  
23 column constructed in accordance with an embodiment of the present invention.

24 FIG. 5 is a chromatogram showing capillary IP-RP-HPLC separation of a mixture  
25 of double-stranded DNA fragments in a monolithic capillary column constructed in  
26 accordance with an embodiment of the present invention. The sample was a pBR322-  
27 *Hae* III digest, 4.5 fmol of each fragment.

28 FIG. 6 is a chromatogram showing capillary IP-RP-HPLC separation of a mixture  
29 of double-stranded DNA fragments in a monolithic capillary column constructed in  
30 accordance with an embodiment of the present invention. The sample was a pBR322-  
31 *Hae* III digest, 1.81 fmol of each fragment.

32 FIG. 7 shows a scanning electron micrograph of underivatized PS-DVB particles.

33 FIG. 8 shows a scanning electron micrograph of octadecylated PS-DVB particles.

34 FIG. 9 shows a scanning electron micrograph of an underivatized PS-DVB  
35 monolith.

1 FIG. 10 illustrates the separation and mass analysis of a series of oligothymidylic  
2 acids.

3 FIG. 11 is a chromatogram showing analysis of a crude synthetic 80-mer  
4 oligodeoxynucleotide by on-line IP-RP-HPLC-ESI-MS.

5 FIG. 12 is a chromatogram showing extraction of selected ion chromatograms  
6 from the data shown in FIG. 11.

7 FIG. 13 is a chromatogram showing averaging and deconvolution of four mass  
8 spectra between 3.7 and 3.8 min from FIG. 11.

9 FIG. 14 is a chromatogram showing the separation and mass analysis of double-  
10 stranded DNA fragments from a *Hae* III digest of pBR322 plasmid (180 fmol of each  
11 fragment).

12 FIG. 15 shows extracted and deconvoluted mass spectra of the 80 pb fragment  
13 of the pBR322 DNA-*Hae* III digest under the same analysis conditions as in FIG. 14.

14 FIG. 16 shows extracted and deconvoluted mass spectra of the 123/124 pb  
15 fragment of the pBR322 DNA-*Hae* III digest under the same analysis conditions as in  
16 FIG. 14.

17 FIG. 17 shows extracted and deconvoluted mass spectra of the 267 pb fragment  
18 of the pBR322 DNA-*Hae* III digest under the same analysis conditions as in FIG. 14.

19 FIG. 18 illustrates IP-RP-HPLC-MS analysis of an unfragmented 5-mer  
20 oligodeoxynucleotide.

21 FIG. 19 shows a mass spectrum obtained from IP-RP-HPLC-ESI-MS/MS  
22 analysis.

### 23 DETAILED DESCRIPTION OF THE INVENTION

24 The present invention generally relates to column chromatography. The  
25 chromatographic separation is carried out by forcing a liquid through a column packed  
26 with a monolithic matrix. A sample, such as a mixture of one or more polynucleotides,  
27 is introduced at the top of the column and then moves with the flow through the column.  
28 The polynucleotides are retarded on the matrix in such a manner that polynucleotides  
29 having different lengths are retarded differently during elution using a mobile phase  
30 gradient of organic solvent.

31 In its most general form, the invention concerns the separation of  
32 polynucleotides. e.g. DNA, utilizing a stationary separation medium having non-polar  
33 surfaces. The separation is performed on the stationary surface. Any surface  
34 micropores preferably are of a size which excludes the smallest polynucleotide being  
35 analyzed. In the invention, the separation surfaces comprise the surfaces of interstitial

1 spaces in a molded polymeric monolith. The preferred separation medium is in the form  
2 of a polymeric monolith such as a monolithic rod. The monolith is polymerized or formed  
3 as a single unit inside of a tube. The channels (i.e., through-pores or macropores)  
4 provide for the passage of eluting solvent and analyte materials. The separation is  
5 performed on the stationary surface. All of the mobile phase is forced to flow through  
6 the channels of the separation medium (Petro et al. *J. Chromatogr. A* 752:59-66  
7 (1996)). Without wishing to be bound by any particular theory, it is believed that mass  
8 transport is enhanced by such convection (Rodrigues et al. *J. Chromatogr.* 653:189  
9 (1993); Liapis, *Math. Modelling Sci. Comput.* 1:397 (1993); Liapis et al. *J. Chromatogr.*  
10 *A* 660:85 (1994)) and has a positive effect on chromatographic efficiency (Afeyan et al.  
11 *J. Chromatogr.* 519:1-29 (1990)).

12 As used herein, the term "non-porous" is defined to include a monolithic  
13 separation surface which has surface micropores having a diameter that is less than the  
14 size and shape of the smallest polynucleotide fragment in the separation in the solvent  
15 medium used therein. Included in this definition are separation surfaces having these  
16 specified maximum size restrictions in their natural state or which have been treated to  
17 reduce their micropore size to meet the maximum effective micropore size required.

18 The surface conformations of monoliths of the present invention can include  
19 depressions and shallow pit-like structures which do not interfere with the separation  
20 process. A pretreatment of a porous monolith to render it non-porous can be effected  
21 with any material which will fill the micropores in the surface of the monolith structure  
22 and which does not significantly interfere with the IP-RP-HPLC process. "IP-RP-HPLC"  
23 includes a process for separating single and double-stranded polynucleotides using a  
24 non-polar separation medium, wherein the process uses a counterion agent, and an  
25 organic solvent to elute the nucleic acid from the non-polar surface of the medium.

26 As used herein, the term "polynucleotide" includes reference to a polymer of  
27 ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), which can be single- or double-  
28 stranded, optionally incorporating synthetic, non-natural, or altered nucleotides capable  
29 of incorporation into DNA or RNA polymers, e.g., methylated nucleotides and nucleotide  
30 analogs. Polynucleotides may have any three-dimensional structure, and may  
31 optionally be partially or fully denatured. The following are non-limiting examples of  
32 polynucleotides: a gene or gene fragment (e.g., restriction fragments), exons, introns,  
33 messenger RNA, transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant  
34 polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any  
35 sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

1 In a general aspect, the invention provides a chromatographic system for separating  
2 a mixture of polynucleotides. The system typically includes a separation column, a  
3 source of mobile phase, a pump, an injector, a column oven, a detector, a fraction  
4 collector, and a computer system including control software.

5 In a preferred embodiment of the instant invention, the chromatographic system  
6 utilizes miniaturized system components and column tubing having small inner  
7 diameters (e.g., having an ID of 1 micrometer to 5,000 micrometer, typically having and  
8 ID of 1 micrometer to 1,000 micrometer, and preferably having a column ID of about 10  
9 micrometer to about 500 micrometer). Four major advantages connected with the use of  
10 smaller dimensions in chromatographic separation techniques can be specified:  
11 increased mass sensitivity with concentration-sensitive detectors allows the analysis of  
12 smaller samples (Novotny); on-line conjugation to mass spectrometry is feasible  
13 (Yergey et al. *Liquid Chromatography/Mass Spectrometry-Techniques and Applications*,  
14 Plenum Press, New York (1990); Niessen et al. *Liquid Chromatography-Mass*  
15 *Spectrometry: Principles and Applications*, Marcel Dekker, Inc., New York, (1992));  
16 higher separation efficiency and better resolving power can be accomplished in shorter  
17 time (Karlsson et al. *Anal. Chem.* 60:1662-1665 (1988)); Kennedy et al. *Anal. Chem.*  
18 61:1128-1135 (1989)); McCloskey in *Mass Spectrometry*, Academic Press Inc., San  
19 Diego (1990)); and expenses connected with consumption of mobile and stationary  
20 phase are cut down.

21 Without wishing to be bound by theory, high efficiency of microcolumns is attributed  
22 to decreased flow dispersion and a very homogenous packing bed structure, in which  
23 the stabilizing influence of the wall is felt by the entire packing bed (Kenndey). The  
24 volume of eluent used in microcolumn chromatography is considerably reduced, which  
25 means that the solutes of interest are dissolved in much less eluent, resulting in higher  
26 mass sensitivity and easier coupling with mass spectrometry.

27 In a preferred embodiment of the instant invention, microcolumn HPLC systems are  
28 designed and operated with the utmost attention to eliminating extracolumn band  
29 dispersion attributable to the sampling volume, detection volume, connecting tubing,  
30 and system time constant (Scott et al. *J. Chromatogr. Sci.* 20:62-66 (1982); Novotny  
31 *Anal. Chem.* 60:500A-510A (1988)). Introduction of small sample volumes and amounts  
32 into microcolumns by direct injection with microinjectors ( $\geq 20$  nL), moving injection  
33 (Borra et al. *J. Chromatogr.* 395:75-85 (1987)), split injection (McGuffin et al. *Anal.*  
34 *Chem.* 55:580-583 (1983)), heart cutting injection (McGuffin et al.), or electrokinetic  
35 injection is mandatory for preventing column overloading and minimizing peak variance.

1 Also in a preferred embodiment of the present invention, the micro-HPLC detector is  
2 miniaturized in order to efficiently detect a narrow peak eluting from a capillary column.  
3 The detector is capable of monitoring the column effluent from capillaries with high  
4 fidelity. An example of a suitable detector is a curved capillary flow cell with improved  
5 performance for capillary HPLC (Chervet et al. *An Improved Method of and a Capillary*  
6 *Flow Cell for Analysing Fluid Samples*, European patent application no. 0597552A1  
7 (1993)). In on-column detection, a section of the capillary column can be converted to  
8 the flow cell upon removing the polyimide coating and is exposed to the light beam of a  
9 conventional UV/VIS spectrophotometric detector (Chen et al. *Anal. Meth. Instr.*, 2:122-  
10 128 (1995)). Other detection methods and ancillary techniques can be used, such as  
11 conductivity, light scattering, evaporative detection, mass spectrometry (Yergey et al.  
12 (1990); Niessen et al. (1992)), electrochemical detection (Colon et al. *Anal. Chem.*  
13 65:476 (1993); Ewing et al. *Anal. Chem.* 66:527A (1994)), radiometric detection (Tracht  
14 et al. *Anal. Chem.* 66:2382 (1994)), and multichannel fluorescence detection  
15 (Timperman et al. *Anal. Chem.* 67:139 (1995)).

16 Because the gradient delay volume must be kept at a minimum, carrying out  
17 gradient elution in miniaturized HPLC is more complicated than using conventional  
18 solvent delivery systems. Some modifications of commercially available solvent delivery  
19 systems include stepwise gradients (Hirata et al. *J. Chromatogr.* 186:521-528 (1979)),  
20 split-flow operation (Van der Wal et al. *J. High Res. Chromatogr.* (1983); Chervet *Micro*  
21 *Flow Processor*, European patent application no. 0495255A1 (1991)), preformed  
22 gradients (Davis et al. *J. Am. Soc. Mass Spectrom.* 6:571-577 (1995)), and miniaturized  
23 diluting chambers (Takeuchi et al. *J. Chromatogr.* 253:41-47 (1982); Karlsson et al. *J.*  
24 *Chromatogr.* 7:411-413 (1984)). Commercially available micro-HPLC instrumentation  
25 with micro-mixing chambers is capable of performing reproducible gradients with flow  
26 rates as low as 5-10  $\mu\text{L}/\text{min}$  without solvent splitting.

27 High pressure pumps are used for pumping mobile phase in the systems described  
28 herein. It will be appreciated that other methods are known for driving mobile phase  
29 through separation media and can be used in carrying out the separations of  
30 polynucleotides as described in the present invention. A non-limiting example of such  
31 an alternative method includes "capillary electrochromatography" (CEC) in which an  
32 electric field is applied across capillary columns packed with microparticles and the  
33 resulting electroosmotic flow acts as a pump for chromatography. Electroosmosis is the  
34 flow of liquid, in contact with a solid surface, under the influence of a tangentially applied  
35 electric field. The technique combines the advantages of the high efficiency obtained

1 with capillary electrophoretic separations, such as capillary zone electrophoresis, and  
2 the general applicability of HPLC. CEC has the capability to drive the mobile phase  
3 through columns packed with chromatographic particles, especially small particles,  
4 when using electroosmotic flow. High efficiencies can be obtained as a result of the  
5 plug-like flow profile. In the use of CEC in the present invention, solvent gradients are  
6 used and rapid separations can be obtained using high electric fields. The following  
7 references describing CEC are each incorporated in their entirety herein: Dadoo, et al,  
8 *LC-GC* 15:630 (1997); Jorgenson, et al., *J. Chromatog.* 218:209 (1981); Pretorius, et  
9 al., *J. Chromatog.* 99:23 (1974); and the following U.S. Patent Nos. to Dadoo 5,378,334  
10 (1995), 5,342,492 (1994), and 5,310,463 (1994). Another example of a method for  
11 driving mobile phase includes centrifugal force, such as described in US 6,063,589.

12 In a particular aspect, the instant invention provides a separation column that  
13 consists of a polymeric monolith having non-polar chromatographic surfaces. The  
14 process for producing the columns generally comprises (1) adding to a rigid tube sealed  
15 at both ends a deaerated polymerizable mixture containing an inert porogen; (2)  
16 polymerizing the mixture, typically in the presence of a catalyst, to form a macroporous  
17 polymer plug; and (3) washing the plug with a solvent so as to remove the porogen  
18 present in the macroporous polymer produced. The polymerizable mixture contains a  
19 suitable monomer or monomer mixture with appropriate amounts of a suitable  
20 crosslinker.

21 Macroporous matrices are obtained when polymerization and crosslinking take  
22 place in the presence of inert porogens which lead to a phase separation during the  
23 ongoing polymerization reaction and effect the formation of permanent channels in the  
24 material (Seidl et al. *Adv. Polymer Sci.*, 5:113-213 (1967); Hjerten et al. *Nature*,  
25 356:810-811 (1992); C. Viklund et al. *Chem. Mater.* 8:744-750 (1996)). The concept of  
26 monolithic stationary phases is especially favorable for the fabrication of capillary  
27 columns.

28 Applicants have found that the exact adjustment of the polymerization conditions  
29 is crucial for the preparation of high performance monoliths of the present invention.  
30 These conditions include use of an inert component, the porogen, or a mixture of such  
31 inert components that do not participate in the polymerization and which are soluble in  
32 or at least miscible with the monomer. Careful control of the polymerization kinetics is  
33 also required to model the morphology of the formed polymer. Temperature, reaction  
34 time, concentration of radical initiator, ratio of monomer to crosslinker affect the  
35 performance of the monolith.

1       The most important parameters for the construction of special channel sizes are  
2 monomer type and reactivity, degree of crosslinking, amount and type of porogen(s),  
3 solvency of the porogen(s) for the polymer, and polymerization temperature (Seidl et al.;  
4 Svec et al. *Macromolecules* 28:7580-7582 (1995); Viklund et al. *Chem. Mater.* 9:463-  
5 471 (1997); Wang et al. *Anal. Chem.* 64:1232-1238 (1992)). To avoid undesired  
6 sedimentation, the columns can be rotated slowly in the course of the polymerization  
7 process. Column permeability and performance can be modulated over a wide range  
8 by varying the amount of porogen in the polymerization mixture. For differing  
9 compositions of the porogen, the amount of radical initiator has to be newly optimized to  
10 maintain a reasonable separation performance. Monoliths with high back pressure can  
11 be obtained using high percentages of porogen, while for columns with lower back  
12 pressure a composition with high amount of initiator and a low percentage of the  
13 porogen tetrahydrofuran is preferred. Additionally, not all the pieces that are cut from  
14 one synthesized capillary monolithic column are identical and the chromatographic  
15 performance of the pieces must be determined

16       The preferred monolithic columns were synthesized to exhibit hydrodynamic  
17 properties comparable to that of packed columns. The back pressure in a 6 cm long  
18 monolithic column (prepared as described in Example 3) for water at a flow rate of 3  
19  $\mu\text{L}/\text{min}$  is was in the range of 90 to 120 bar, which compares well to a column packed  
20 with non-porous beads of equal dimensions and comparable chromatographic efficiency  
21 that exhibited a back pressure of 150 bar. The lower back pressure in monoliths is a  
22 result of increased macroporosity. The monoliths of the invention can be used at back  
23 pressures in the range of about 20 to 300 bar. The back pressure will be dependant  
24 upon the dimensions, the length and inner diameter, of the tube. In general, a shorter  
25 tube will give a lower back pressure.

26       The method preferably is performed at an elution temperature within the range of  
27 20°C to 90°C.

28       In an important aspect, the instant invention is based on the surprising and  
29 unexpected discovery that an underivatized poly(styrene-divinylbenzene) (PS-DVB)  
30 monolith exhibited highly efficient separation performance. This was unexpected, since  
31 the disclosure in the published patent application WO 00/15778, which further cited  
32 other suggestions in the literature, disclosed that underivatized  
33 poly(polystyrene/divinylbenzene) structures are not desirable for DNA separations. It  
34 was disclosed that no useful separation using such monoliths were obtained. In the

1 present invention, the term "underivatized", as used in describing a monolithic matrix, is  
2 used herein to indicate that the monolithic matrix is not substituted with alkyl moieties  
3 (such as straight chain, branched or aromatic hydrocarbons) or with non-alkyl moieties  
4 (such as charged or polar groups).

5 In preparing the monoliths of the present invention, a preferred monomer is styrene  
6 and a preferred crosslinking agent is divinylbenzene. Examples of preferred porogens  
7 include toluene, decanol, hexane and tetrahydrofuran.

8 Based on preliminary experiments, a ratio of monomer to porogen mixture of 2:3  
9 was found suitable in the preparation of the monoliths of the present invention. The  
10 chemical purity of the commercially available styrene was better than 99%. However,  
11 an assay of the utilized divinylbenzene revealed that only 65% of the used reagent were  
12 indeed isomers of divinylbenzene, capable of performing the crosslinking of polymer  
13 chains, while a percentage of about 33% was formed by different ethylvinyl benzenes  
14 that can act as a monomer for polymerization, but not crosslinker. In the description  
15 herein, the true amount or percentage of chemically pure divinylbenzene is indicated,  
16 and the amount or percentage of the non crosslinking ethylvinyl benzene was added to  
17 that of styrene. The composition of mixtures is either given in absolute masses or as  
18 percentages weight-per-weight. The density of the most used reagents is given in Table  
19 1.

20 Table 1

21 Density of the components of the polymerization mixture

| <i>component</i> | <i>density <math>\tau_{20^{\circ}\text{C}}</math> [kg/m<sup>3</sup>]</i> |
|------------------|--------------------------------------------------------------------------|
| styrene          | 909                                                                      |
| divinylbenzene   | 914                                                                      |
| 1-decanol        | 829                                                                      |
| hexane           | 660                                                                      |
| tetrahydrofuran  | 889                                                                      |
| toluene          | 867                                                                      |

22 In a preferred embodiment of the instant invention, the monolith is comprised of an  
23 underivatized poly(styrene-divinylbenzene) matrix. Applicants have surprisingly  
24 discovered that the porogenic solvent tetrahydrofuran gave monolithic columns  
25 displaying unexpectedly high efficiency of separation of polynucleotides. Therefore, a

1 preferred porogenic solvent includes tetrahydrofuran. A more preferred porogen solvent  
2 comprises a mixture of tetrahydrofuran and decanol.

3 An embodiment of a polymerization mixture for the synthesis of suitable columns for  
4 the separation of biopolymers included the following: 0.5948 g non-crosslinking  
5 monomer (styrene + ethylvinyl benzene), 0.2911 g crosslinker (divinylbenzene), 1.0062  
6 g 1-decanol, 0.1759 g tetrahydrofuran, and 0.0193 g  $\alpha,\alpha'$ -azobisisobutyronitrile (ABIN).  
7 Monolithic capillary columns were produced by polymerization at 70°C for 24 hours.

8 Without wishing to be bound by theory, it is believed that the improved separation  
9 performance of the monolithic columns of the instant invention is due to the use of  
10 tetrahydrofuran as microporgen, which is more polar and of poorer solvency for the  
11 polymer than the commonly used toluene. The resulting polymer contains relatively  
12 large channels that allow rapid convective mass transport between the mobile phase  
13 and a thin out layer of the polymer. This configuration adequately imitates the  
14 configuration of micropellicular, beaded stationary phases (e.g., as disclosed in US  
15 5,585,236), which have been shown to be highly suitable for high-speed separations of  
16 biopolymers.

17 In another aspect, the instant invention provides a monolith that is contained  
18 within a tube and which is immobilized at the inner wall of the tube. In a preferred  
19 embodiment, the monolith is immobilized by covalent attachment at the inner wall of the  
20 tube. Shrinking of the monolith can be an issue during polymerization. The overall  
21 volume shrinkage during polymerization of methacrylate polymers amounts to  
22 approximately 6%, and shrinkage occurs mainly at a late point in polymerization within  
23 the formed and already crosslinked monolith (Brooks *Macromol. Chem., Macromol.*  
24 *Symp.* 35/36:121 (1990)). Therefore an extension of the channels is to be expected  
25 rather than a shrinkage of the exterior dimensions and detaching from the capillary wall.  
26 Furthermore, derivatization of the capillary inner wall with vinylsilanes facilitates wetting  
27 with polymerization mixture, reduces the formation of bubbles and can be used to  
28 chemically attach the formed polymer to the silica surface. A representative example of  
29 a process for covalent attachment is shown in FIG. 1 and as described in Example 2.

30 Monoliths that were prepared without previous vinylsilylation, and thus without  
31 anchoring to the inner capillary wall, underwent compression when subjected to high  
32 pressures from a HPLC pump. A monolith was prepared by allowing to react a mixture  
33 of 0.40 mL styrene, 1.80 mL divinylbenzene, 50 mg AIBN, 2.25 mL 1-decanol and 0.75  
34 mL toluene at 70°C for 14 hours within a 200 × 0.32 mm fused silica capillary.  
35 Acetonitrile was pumped through an 80 mm long piece of this capillary and the monolith

1 was held in its place by a PEEK frit in a stainless steel union. A longitudinal  
2 compression of 2 mm out of 80 mm, corresponding to a reduction of length of 2.5%,  
3 was observed when a pressure of 200 bar was applied. The pressure-to-flow curve  
4 starts out linearly, but begins to rise exponentially as soon as compression of the  
5 monolith and thus restriction of the channels begins at a flow rate of 6  $\mu\text{L}/\text{min}$  and a  
6 respective back pressure of 25 bar (data not shown). No such compression was  
7 observed and a linear pressure-to-flow curve over the whole range of tested flow rates  
8 and applied pressure was observed with monoliths that were chemically immobilized to  
9 the surface.

10 The monolithic columns prepared as described herein can be equipped with  
11 conventional retaining frits. However, in preferred embodiments, the monolithic  
12 columns of the invention are devoid of retaining frits. Thus in another aspect, the  
13 invention provides a polymeric monolith, preferably an underivatized poly(styrene-  
14 divinylbenzene) monolith, that is contained within a tube wherein the tube is devoid of  
15 retaining frits. In preferred embodiments, the monolith is immobilized at the capillary  
16 wall during polymerization. Such immobilization eliminates the necessity to prepare a  
17 tiny retaining frit, which is one of the more tedious and difficult to control steps during  
18 the manufacture of packed bed capillary columns (Svec et al. *Macromolecules* 28:7580-  
19 7582 (1995); C. Ericson et al. *J. Chromatogr. A* 767:33-41 (1997); Oberacher et al. *J.*  
20 *Chromatogr. A* 893:23-35 (2000)). Capillary columns prepared without frits are thus  
21 easier to prepare and less expensive.

22 After polymerization is complete, the solid monolith is preferably washed to  
23 remove any porogenic solvent and with a suitable solvent to dissolve any soluble  
24 polymer present. Suitable washing solvents include methanol, ethanol, benzene,  
25 acetonitrile, toluene acetone, tetrahydrofuran, and dioxane. This washing process may  
26 be done in stages; for example by alternatively washing with solvent and water, or by  
27 continuous washing with a solvent. The washing step is performed by pumping the  
28 solvent through the tube filled with the monolith.

29 A wide variety of conventional support structures, such as a tube, a channel or  
30 groove on a plate, a thin film across a plate, or a microchip, can be used with the  
31 monolithic matrix of the instant invention. Examples of such structures are described,  
32 for example, in WO 00/15778.

33 A still further aspect of the instant invention concerns the morphology of the  
34 surface structure of the monolith. The morphology of the synthesized monolithic  
35 polymers was optically characterized by light microscopy and by scanning electron

1 microscopy. The homogeneity of the monolithic stationary phase over the length of the  
2 capillary was controlled using an Olympus BH-2 light microscope (magnification factor  
3 from 40 to 1,000). Electron micrographs were acquired using a Voyager ARL-SEM-Q-  
4 electron micrograph (Noran Instruments Inc., Middleton, WI) with a magnification factor  
5 from 200 to 30,000.

6 Scanning electron micrographs were acquired to characterize the column  
7 morphology and surface structure. FIG. 9 shows scanning electron micrographs of the  
8 stationary phase made of a highly crosslinked, underivatized, styrene-divinylbenzene  
9 copolymer monolith. The cross section of the rod reveals clusters of globules separated  
10 by large channels. The average size of the globules is in the range of 100 to 200 nm,  
11 they form the building units for larger aggregates with a diameter from 500 to 800 nm.  
12 The size of the channels between the clusters reaches 500 nm, corresponding well to  
13 those measured by inverse size exclusion chromatography.

14 The surface structure of the poly(styrene-divinylbenzene) monoliths of the present  
15 invention was compared to that of octadecyl modified poly(styrene-divinylbenzene)  
16 particles, which have been shown to be highly suitable for high-speed separation of  
17 polynucleotides (Huber et al. *Anal. Biochem.* 212:351-358 (1993); US 5,585,236). The  
18 monolith was observed to have relatively large channels. Without wishing to be bound  
19 by theory, these channels are thought to allow rapid convective mass transport between  
20 the mobile phase and a thin outer layer of the polymer.

21 Applicants surprisingly discovered that the surface structure of the underivatized  
22 poly(styrene-divinylbenzene) monoliths resembled the surface structure of the octadecyl  
23 derivatized beads, both showing a surface that appeared rugulose, but not the  
24 underivatized beads, which showed a smooth surface. While derivatization with  
25 octadecyl groups has been shown to be essential to obtain high chromatographic  
26 efficiency with PS-DVB particles (Huber et al. *Anal. Biochem.* 212:351-358 (1993);  
27 Huber et al. *Nucleic Acids Res.* 21:1061-1066 (1993)) monolithic stationary phases  
28 exhibited superior efficiency already without derivatization. Without wishing to be bound  
29 by theory, one possible explanation for this different behavior is the formation of the  
30 polymer in two different chemical environments. The PS-DVB particles were  
31 polymerized in aqueous suspension, where poor solvation of the hydrophobic polymer  
32 by the hydrophilic solvent resulted in a relatively flat surface, as revealed by the  
33 scanning electron micrograph depicted in FIG. 7. Particles that were derivatized with  
34 octadecyl groups showed a rugulose surface (FIG. 8) possibly offering a contact area  
35 greater than that of a smooth spherical particle. The formation of the monolithic bed, on

1 the other hand, took place in an entirely organic environment. During polymerization,  
2 small primary particles of approximately 0.5  $\mu\text{m}$  coagulated to form the porous monolith,  
3 resulting in a surface structure (FIG. 9) that resembled the rugulose surface of the  
4 octadecylated PS-DVB particles (FIG. 8). Without wishing to be bound by theory,  
5 Applicants believe that the very rugulose surface of the stationary phase of the monolith  
6 of the present invention offers a contact area greater than that of smooth spherical  
7 particles and that this enhanced contact area gives improved separation performance. A  
8 "rugulose surface" as defined herein includes a surface characterized by showing many  
9 small wrinkles. It was also observed that a particle that was derivatized with octadecyl  
10 groups had a brush-like surface (FIG. 8). A "brush-like surface" as defined herein  
11 includes a surface characterized by showing many small bristles on the surface. The  
12 monolith (FIG. 9) also had a brush-like surface structure, unlike the underivatized  
13 particle (FIG. 7).

14 In still another aspect, the poly(styrene-divinylbenzene) monolith of the present  
15 invention provides a non-porous chromatographic surface. With a gradient of 4.0-  
16 12.0% acetonitrile in 50 mM TEAA in 10 min, oligothymidylic acids as small as the 3-  
17 mer were eluted as sharp and symmetric peaks (chromatogram not shown). From the  
18 crystal structure of the trinucleotide ( $\text{A}$ )<sub>3</sub> it can be inferred, that a 3-mer  
19 oligodeoxynucleotide has an almost globular structure with a diameter of approximately  
20 1.0 nm (Suck et al. *Acta Crystallogr., Sect. B* 32:1727-1737 (1976)). Because  
21 penetration of analytes into micropores of commensurate size would cause  
22 considerable band broadening, the capability of the monolithic stationary phase to  
23 efficiently separate such small molecules is a good indicator for the absence of  
24 micropores.

25 A still further aspect of the present invention is based on the surprising discovery  
26 that the underivatized poly(styrene-divinylbenzene) monoliths having nonpolar  
27 chromatographic surfaces were found to provide unusually high efficiency of separation  
28 of polynucleotides. In this aspect, the invention provides a monolith characterized by  
29 having high separation efficiency as indicated by a high number of theoretical plates per  
30 meter. Two terms are widely used as quantitative measures of band spreading and  
31 thus chromatographic column efficiency: the plate height  $H$  and the number of  
32 theoretical plates  $N$ . The two parameters are related by the equation:

$$N = L/H \quad (1)$$

The plate height and the dimensionless number of theoretical plates express the peak variance per unit length of the column and the dimensionless peak variance, respectively (Poole et al. *Chromatography Today*, Elsevier, Amsterdam (1995); *Practical HPLC Method Development* Snyder et al. Eds., John Wiley & Sons, New York, pp. 40-47 (1997)). Assuming that the form of the chromatographic peak can be approximated by a Gaussian curve, the number of theoretical plates can experimentally be determined from the equation:

$$N = 5.54 \left( \frac{t_R}{w_{0.5}} \right)^2 \quad (2)$$

$t_R$ ..... retention time [sec]

$w_{0.5}$ .... peak width at half height [sec]

The number of theoretical plates and the plate height are widely used in the art as measures of column performance. For these numbers to be meaningful in comparing two columns, it is essential that they are determined with the same compound and under the same isocratic elution conditions.

In a preferred embodiment of this aspect of the present invention, calculation of the number of theoretical plates is based on the retention time of a single polynucleotide standard under isocratic conditions. A preferred standard comprises a single-stranded oligodeoxynucleotide. In one example, the single-stranded polynucleotide, poly(dT)<sub>18</sub> was used as a standard for the determination of the number of theoretical plates per meter. The chromatographic efficiency of the monolithic columns was determined by isocratic elution of poly(dT)<sub>18</sub> with a mobile phase containing 7.8% acetonitrile in 100 mM TEAA at a flow rate of 2.4  $\mu$ L/min. At 50°C column temperature, the number of theoretical plates exceeded 11,500 plates for a 60 mm column, corresponding to (N/L)= 191,000 theoretical plates per meter.

The capillary monolithic column of the present invention is characterized by having in the range of between about 10,000 and about 200,000 theoretical plates per meter, preferably between 100,000 and 200,000 theoretical plates per meter, more preferably at least 100,000 plates per meter, and most preferably at least 190,000 theoretical plates per meter. Without wishing to be bound by theory, it is believed that one of the main reasons for the high separation efficiency of the monoliths is the rapid mass transfer with the only particle-based diffusion limitation in a thin layer at the surface of monolith.

1 In another aspect, the invention provides a method for separating a mixture of  
2 polynucleotides in which the method includes applying the mixture of polynucleotides to  
3 a polymeric monolith, such as an underivatized poly(styrene-divinylbenzene) monolith,  
4 having non-polar chromatographic surfaces and eluting the mixture of polynucleotides  
5 with a mobile phase comprising a counterion agent and an organic solvent. When  
6 analyzing double-stranded polynucleotides, the method can be used to analyze  
7 polynucleotides having a wide range of lengths. For example, the method can be used  
8 in analyzing polynucleotides having lengths in the range of about 3 base pairs to about  
9 600 base pairs. The method can also be used in analyzing polynucleotides having up  
10 to about 2,000 base pairs. The elution step preferably uses a mobile phase containing  
11 a counterion agent and a water-soluble organic solvent. Examples of a suitable organic  
12 solvent include alcohol, acetonitrile, dimethylformamide, tetrahydrofuran, ester, ether,  
13 and mixtures of one or more thereof, e.g., methanol, ethanol, 2-propanol, 1-propanol,  
14 tetrahydrofuran, ethyl acetate, acetonitrile. The counterion agent is preferably selected  
15 from the group consisting of lower alkyl primary amine, lower alkyl secondary amine,  
16 lower alkyl tertiary amine, lower trialkylammonium salt, quaternary ammonium salt, and  
17 mixtures of one or more thereof. Examples of suitable counterion agents include  
18 triethylammonium acetate (TEAA) and triethylammonium bicarbonate (TEAB).

19 In an additional aspect, the invention provides a method for separating a mixture  
20 of polynucleotides in which the method includes applying the mixture of polynucleotides  
21 to a poly(styrene-divinylbenzene) monolith, such as underivatized poly(styrene-  
22 divinylbenzene), having non-polar chromatographic surfaces and eluting the mixture of  
23 polynucleotides with a mobile phase comprising a counterion agent and an organic  
24 solvent, wherein the mobile phase is devoid of metal chelating agent, such as EDTA.  
25 The elutions described in the Examples herein are performed using mobile phase  
26 lacking EDTA. Avoiding the use of EDTA is an advantage since EDTA in eluted  
27 fractions can interfere with subsequent mass spectral analysis. Removal of EDTA  
28 would require additional processing steps.

29 In a still further aspect, the invention concerns a method for separating a mixture  
30 of polynucleotides in which the method includes applying the mixture of polynucleotides  
31 to a poly(styrene-divinylbenzene) monolith having non-polar chromatographic surfaces  
32 and eluting the mixture of polynucleotides with a mobile phase comprising a counterion  
33 agent and an organic solvent, in which the method further includes analyzing the eluted  
34 polynucleotides by mass spectral analysis. The monolithic column can be operatively  
35 coupled to a mass spectrometer for determining the molecular mass of the eluted

1 polynucleotides. In a preferred embodiment, the mass spectrometer comprises an  
2 electrospray ionization (ESI) mass spectrometer. The electrospray ionization mass  
3 spectrometer can include a tandem mass spectrometer for determining the base  
4 sequences of the polynucleotides.

5 The possibility of direct on-line conjugation of capillary HPLC to mass  
6 spectrometry makes available highly valuable information about the structure and  
7 identity of the separated compounds (Tomer et al. *Mass Spectrom. Rev.* 13:431-457  
8 (1994)). Electrospray ionization mass spectrometry (ESI-MS), by virtue of the multiple  
9 charging of biopolymers and the very soft ionization process, has become one of the  
10 most important mass spectrometric techniques for the analysis of nucleic acids  
11 (Nordhoff et al. *P. Mass Spectrom. Rev.* 15:76-138 (1996)). Nevertheless, the success  
12 of ESI-MS for the characterization of nucleic acids largely depends on the purity of the  
13 sample that is introduced into the mass spectrometer (Portier et al. *Nucleic Acids Res.*  
14 22:3895-3903 (1994)). The major difficulties arise due to the tendency of nucleic acids  
15 to form quite stable adducts with cations resulting in mass spectra of poor quality (Stults  
16 et al. *Rapid Commun. Mass Spectrom.* 5:359-363 (1991); Huber et al. *Anal. Chem.*  
17 70:5288-5295 (1998)). As described hereinbelow, Applicants have observed that the  
18 on-line sample preparation of polynucleotides by chromatographic separation prior to  
19 ESI-MS removes cations from nucleic acid samples, and can be used to fractionate the  
20 polynucleotides in mixtures that are too complex for direct infusion ESI-MS.  
21 The potential to obtain high quality ESI-mass spectra of large, double-stranded DNA is  
22 essentially determined by the amount of salt as well as the number of different  
23 compounds present in the sample mixture (Portier et al. *Nucleic Acids Res.* 22:3895-  
24 3903 (1994); Muddiman et al. *Anal. Chem.* 68:3705-3712 (1996)). Recently, Muddiman  
25 et. al. published the mass spectrum of a 500 bp polymerase chain reaction product,  
26 which has been purified by ethanol precipitation followed by microdialysis (Muddiman et  
27 al. *Rapid Commun. Mass Spectrom.* 13:1201-1204 (1999)). Although the amount of  
28 DNA that was analyzed in the ion cyclotron resonance mass spectrometer was in the  
29 low femtomol range, much more material was required for purification before mass  
30 measurement. Hence, there is an urgent need for rapid on-line separation and  
31 purification protocols requiring only minute sample amounts.

32 In a yet further aspect, the present invention provides a method for desalting and  
33 separating a mixture of single-stranded polynucleotides. The method includes dissolving  
34 a mixture of single-stranded polynucleotides in a mobile phase having a lower  
35 concentration of organic solvent than an initial mobile phase composition. The method

1 further includes loading the mixture onto a poly(styrene-divinylbenzene) monolithic  
2 column, as described herein, and flowing initial mobile phase containing a counterion  
3 agent and having a concentration of organic solvent that is below the level that would  
4 elute the polynucleotides through the column such that the polynucleotides are retained  
5 and the salts are removed from the polynucleotides. The method further includes  
6 separating the mixture of polynucleotides by eluting the mixture of polynucleotides with  
7 a mobile phase comprising a counterion agent and an organic solvent. This desalting  
8 method preferably includes preconcentrating the polynucleotides on the monolithic  
9 column. The volume loading capacity describes the maximum injection volume at  
10 constant analyte amount that can be loaded onto a separation column without the  
11 occurrence of peak broadening. Analytes which are present in extremely low  
12 concentrations in the sample may necessitate the injection of large sample volumes.  
13 Biomolecules exhibit very steep capacity curves in the reversed-phase mode and react  
14 very sensitive to small changes in mobile phase composition. Hence, a  
15 preconcentration at the column head occurs and injection of large volumes of sample  
16 containing a low concentration of analyte is possible without deleterious effects on the  
17 separation efficiency.

18 Monolithic capillary columns as described herein have numerous advantages  
19 when used in the separation of polynucleotides. The preparation can be carried out  
20 following simple procedures and an improved chromatographic separation performance  
21 can be obtained. Specific advantages include:

- 22 - The small volumes and low amounts of samples available from biochemical, medical  
23 or molecular biological experiments are most adequately processed by micro separation  
24 techniques.
- 25 - Polymerization within the confines of fused silica capillaries of small inner diameter is a  
26 straightforward way to manufacture monolithic columns for capillary and nano HPLC.
- 27 - By anchoring the chromatographic support material to the capillary wall using covalent  
28 chemical bonding, no tedious preparation of frits is necessary. Moreover, there is no  
29 need to pack columns using high pressure devices and no restrictions in achievable  
30 capillary length apply.
- 31 - The permeability of the monolithic capillary columns can be modulated by choosing an  
32 appropriate polymerization mixture. Columns with high permeability exhibit a lower back  
33 pressure than packed capillary columns and greater capillary lengths are possible for  
34 chromatographic separations.

- 1 - The enhanced mass transport through continuous macroporous polymer has a  
2 positive effect on chromatographic efficiency.  
3 - Expenses connected with consumption and disposal of materials are cut down.  
4 -The low flow rates applied in microcolumn high performance liquid chromatography are  
5 ideally suited for on-line coupling with electrospray ionization mass spectrometry.

6 Other features of the invention will become apparent in the course of the  
7 following descriptions of exemplary embodiments which are given for illustration of the  
8 invention and are not intended to be limiting thereof.

9 Procedures described in the past tense in the Examples below have been carried  
10 out in the laboratory. Procedures described in the present tense have not yet been  
11 carried out in the laboratory, and are constructively reduced to practice with the filing of  
12 this application.

#### 14 Example 1

##### 15 *Chemicals and oligodeoxynucleotide samples*

16 Acetonitrile (HPLC gradient-grade), divinylbenzene (synthesis grade), methanol  
17 (HPLC gradient-grade), styrene (synthesis grade), and tetrahydrofuran (analytical  
18 reagent grade) were obtained from Merck (Darmstadt, Germany). Styrene and  
19 divinylbenzene were distilled before use. Acetic acid (analytical reagent grade),  
20 azobisisobutyronitrile (synthesis grade), decanol (synthesis grade), and triethylamine  
21 (p.a.) were purchased from Fluka (Buchs, Switzerland). A 1.0 M stock solution of  
22 triethylammonium acetate (TEAA) was prepared by dissolving equimolar amounts of  
23 triethylamine and acetic acid in water. A 0.50 M stock solution of triethylammonium  
24 bicarbonate (TEAB) was prepared by passing carbon dioxide gas (AGA, Vienna,  
25 Austria) through a 0.50 M aqueous solution of triethylamine at 5°C until pH 8.4-8.9 was  
26 reached. For preparation of all aqueous solutions, high-purity water (Epure, Barnstead  
27 Co., Newton, MA, USA) was used. The standards of phosphorylated and non-  
28 phosphorylated oligodeoxynucleotides ((dT)<sub>12-18</sub>, p(dT)<sub>12-18</sub>, p(dT)<sub>19-24</sub>, p(dT)<sub>25-30</sub>) were  
29 purchased as sodium salts from Pharmacia (Uppsala, Sweden) or Sigma-Aldrich (St.  
30 Louis, MO, USA). The synthetic oligodeoxynucleotides (dT)<sub>24</sub> (M<sub>r</sub> 7,238.71), a 5'-  
31 dimethoxytritylated 5-mer (DMTr-ATGCG, M<sub>r</sub> 1805.42), and an 80-mer (M<sub>r</sub> 24,527.17):  
32 CCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCA  
33 GCAATAAACCAGCCAGCCGGAAGGG (SEQ ID NO:1)

1 were ordered from Microsynth (Balgach, Switzerland) and used without further  
2 purification. The size standard of double-stranded DNA restriction fragments (pBR322  
3 DNA-Hae III digest) was purchased from Sigma Aldrich.

#### 4 Example 2

##### 5 *Preparation of fused silica capillaries*

6 Fused silica capillaries with an inner diameter of 200  $\mu\text{m}$  and a length of 3 m were  
7 flushed with 2 mL of methanol and 2 mL of water, filled with 1 mol/L sodium hydroxide,  
8 closed at the ends and allowed to stand for 10 min at room temperature. Subsequently,  
9 the capillary was washed with 2 mL of water and 2 mL of methanol, and dried with  
10 nitrogen for 15 min at room temperature. Before *in situ* polymerization the inner wall of  
11 the fused silica tube was silanized in order to facilitate wetting by the solution of the  
12 monomer mixture and to allow covalent immobilization of the monolith in the tube  
13 (FIG.1). By attaching the bed to the tubing wall, gap formation between the capillary wall  
14 and the polymer due to shrinking of the polymer upon a change of solvent is avoided  
15 and no frit to support the bed is required.

16 In the silanization process, a mixture of 50% (v/v) 3-(trimethoxysilyl)propyl  
17 methacrylate and 0.01% (w/v) 2,2-diphenyl-1-picrylhydrazyl hydrate in  
18 dimethylformamide (DMF) was degassed with nitrogen for 5 min and filled into a  
19 pretreated, 3 m piece of fused silica capillary tubing (Huang et al. *J. Chromatogr. A*  
20 788:155-164 (1997)). The ends of the tubing were closed with silicon stoppers and the  
21 capillary was kept in an oven at 120°C for six hours. Next the capillary was flushed with  
22 2 mL each of DMF, methanol and dichloromethane, and finally dried with nitrogen.

#### 23 Example 3

##### 24 *Preparation of continuous-bed and packed-bed capillary columns*

25 Polyimide coated fused silica capillary tubing of 350  $\mu\text{m}$  OD and 200  $\mu\text{m}$  ID was  
26 obtained from Polymicro Technologies (Phoenix, AZ, USA). A 1 m piece of fused silica  
27 capillary tubing was silanized with 3-(trimethoxysilyl)propyl methacrylate (Huang et al.  
28 *C. J. Chromatogr. A* 788:155-164 (1997)) in order to ensure immobilization of the  
29 monolith at the capillary wall. Then, a 300 mm piece of the silanized capillary was filled  
30 with a mixture comprising 50  $\mu\text{L}$  styrene, 50  $\mu\text{L}$  divinylbenzene, 130  $\mu\text{L}$  decanol, 20  $\mu\text{L}$   
31 tetrahydrofuran, and 10 mg/mL azobisisobutyronitrile with a plastic syringe. The mixture  
32 was polymerized at 70 °C for 24 hours. After polymerization, the capillary was  
33 extensively flushed with acetonitrile at a flow rate of 5.0  $\mu\text{L}/\text{min}$  and finally cut into 60  
34 mm long pieces. Octadecylated PS-DVB particles (PS-DVB-C<sub>18</sub>) were synthesized as

published in the literature (Huber et al. *Anal. Biochem.* 212:351-358 (1993)). The PS-DVB-C<sub>18</sub> stationary phase has been commercialized as DNASep® columns by Transgenomic Inc. (San Jose, CA, USA). Packed-bed capillary columns were prepared according to the procedure described (Oberacher et al. *J. Chromatogr. A* (2000)).

#### Example 4

##### *High-performance liquid chromatography*

The HPLC system consisted of a low-pressure gradient micro pump (model Rheos 2000, Flux Instruments, Karlskoga, Sweden) controlled by a personal computer, a vacuum degasser (Knauer, Berlin, Germany), a column thermostat made from 3.3 mm OD copper tubing which was heated by means of a circulating water bath (model K 20 KP, Lauda, Lauda-Königshofen, Germany), a microinjector (model C4-1004, Valco Instruments Co. Inc., Houston, TX, USA) with a 200 or 500 nL internal sample loop, a variable wavelength detector (model UltiMate UV detector, LC Packings, Amsterdam, Netherlands) with a Z-shaped capillary detector cell (part no. ULT-UC-N-10, 3nL cell, LC Packings), and a PC-based data system (Chromeleon 4.30, Dionex-Softron, Germering, Germany).

#### Example 5

##### *High-resolution capillary IP-RP-HPLC separation of phosphorylated oligodeoxynucleotide ladders in a monolithic capillary column*

Using the column as described in Example 3, a high-resolution capillary IP-RP-HPLC separation of a phosphorylated oligodeoxynucleotide ladder was performed (FIG. 2): Column, continuous PS-DVB, 80 × 0.20 mm ID; mobile phase, buffer A 100 mM TEAA, pH 6.80, buffer B 100 mM TEAA, pH 6.80, 20% acetonitrile; linear gradient, 32-42% B in 3.0 min, 42-52% B in 7 min; flow-rate, 3.3 µL/min; temperature, 50 °C; detection, UV, 254 nm; sample, p(dT)<sub>12-30</sub>, 6 ngram each/0.66 - 1.64 pmol each.

#### Example 6

##### *Separation of phosphorylated oligodeoxyadenylic- and oligothymidylic acids*

FIG. 3 illustrates the high-resolution separation of phosphorylated oligodeoxyadenylic- and oligothymidylic acids ranging in size from 12-30 nt. Gradient elution with 3.0-9.0% acetonitrile in 3.5 min, followed by 9.0-11.0% acetonitrile in 2.5, and finally 11.0-13.0% acetonitrile in 4.0 min in 100 mM TEAA resulted in peak widths at half height of 1.3 s for p(dA)<sub>12</sub> to 2.4 s for p(dT)<sub>30</sub> which allowed the baseline resolution of the whole series up to the 30-mer within 8.2 min. The resolution of homologous oligodeoxynucleotides obtained with the monolithic column clearly

surpasses that of a capillary column packed with PS-DVB-C<sub>18</sub> beads (Table 2, compare also Figure 1 in Huber et al. *Anal. Chem.* 71:3730-3739 (1999)).

**Table 2**

**Comparison of the Resolution Values for Oligodeoxynucleotides and Double-stranded DNA using Packed and Monolithic Capillary Columns**

| compounds                                | resolution with packed column | resolution with monolithic column |
|------------------------------------------|-------------------------------|-----------------------------------|
| p(dT) <sub>12</sub> /p(dT) <sub>13</sub> | 3.05                          | 5.38                              |
| p(dT) <sub>29</sub> /p(dT) <sub>30</sub> | 1.04                          | 2.38                              |
| 51/57 bp                                 | 3.88                          | 5.15                              |
| 540/587 bp                               | 1.11                          | 2.70                              |

In this example the high-resolution capillary IP-RP-HPLC separation of phosphorylated oligodeoxynucleotide ladders was performed using a monolithic capillary column: Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A included 100 mM TEAA, pH 6.97, buffer B included 100 mM TEAA, pH 6.97, 20% acetonitrile; linear gradient, 15-45% B in 3.5 min, 45-55 % B in 2.5 min, 55-65 % B in 4.0 min; flow-rate, 2.5 µL/min; temperature, 50 °C; detection, UV, 254 nm; sample, p(dA)<sub>12-18</sub>, p(dT)<sub>12-30</sub>, 40 - 98 fmol of each oligodeoxynucleotide.

**Example 7**

*Performance of monolithic capillary columns for polynucleotide separations*

Following polymerization, extensive washing with acetonitrile, and equilibration with 100 mM TEAA-5.0% acetonitrile solution, the performance of three different 60 x 0.20 mm ID monolithic capillary columns was compared to that of three columns packed with octadecylated, 2.3 µm micropellicular PS-DVB particles of the same dimensions. The permeabilities of the monolithic columns and the packed columns were similar resulting in back pressures between 180 and 200 bar at a flow rate of 2.6 µL/min and 50°C column temperature, which indicates that the size of the channels for convective flow in both chromatographic beds is of approximately the same size. The relative standard deviations of the peak widths at half height both among various batches of packed capillary columns and monolithic capillary columns were better than 10% which demonstrates that column preparation was reproducible and allowed the comparison of the chromatographic performance of both column types. The chromatographic performance was evaluated by gradient separation of a mixture of (dT)<sub>12-18</sub> with a

1 gradient of 5.0-12.0% acetonitrile in 100 mM TEAA in 10 min. Three injections of the  
2 standard onto each of the three columns gave average peak widths at half height for  
3  $(dT)_{18}$  of  $2.28 \pm 0.22$  s (sample size  $N=9$ , standard deviation  $sd=0.29$  s, level of  
4 significance  $P=95\%$ ) for the monolithic columns and  $3.84 \pm 0.16$  s ( $N=9$ ,  $sd=0.20$  s,  
5  $P=95\%$ ) for the packed bed capillary column. These values demonstrate that the  
6 chromatographic performance of monolithic columns for oligodeoxynucleotide  
7 separations is approximately 40% better than that of packed bed columns. The  
8 chromatographic efficiency of the monolithic columns was determined by isocratic  
9 elution of  $(dT)_{18}$  with an eluent containing 7.8% acetonitrile in 100 mM TEAA at a flow  
10 rate of  $2.4 \mu\text{L}/\text{min}$ . At  $50^\circ\text{C}$  column temperature, the number of theoretical plates  
11 exceeded 11,500 plates for a 60 mm column, corresponding to 191,000 theoretical  
12 plates per meter.

### 13 Example 8

#### 14 *High-resolution capillary IP-RP-HPLC separation of a mixture of phosphorylated and* 15 *dephosphorylated deoxyadenylic acids*

16 The separation shown in FIG. 4 was performed under the following condition:  
17 Column, monolithic PS-DVB, 60 mm x 0.20 mm ID; mobile phase, buffer A included 100  
18 mM TEAA, pH 7.00, buffer B included 100 mM TEAA, pH 7.00, 20% acetonitrile; linear  
19 gradient, 5-30% B in 5.0 min, 35-40 % B in 5.0 min, 40-45 % B in 6.0 min, 45-52 % B in  
20 14 min; flow-rate,  $2.1 \mu\text{L}/\text{min}$ ; temperature,  $50^\circ\text{C}$ ; detection, UV, 254 nm; sample,  
21 hydrolyzed  $p(dA)_{40}$  -  $p(dA)_{60}$ , spiked with 2.5 ng  $p(dA)_{12}$  -  $p(dA)_{18}$ .

### 22 Example 9

#### 23 *Separation of double-stranded DNA using a PS-DVB monolithic column*

24 IP-RP-HPLC has been shown to be efficient not only for the rapid separation of  
25 single-stranded oligodeoxynucleotides, but also for the fractionation of double-stranded  
26 DNA fragments up to chain lengths of 2000 bp (Huber et al. *Anal. Chem.* 67:578-585  
27 (1995)). The applicability of the monolithic PS-DVB stationary phase to the IP-RP-HPLC  
28 separation of double-stranded DNA was tested by injection of a pBR322 DNA-*Hae* III  
29 digest, which was separated in 12.5 min using a gradient of 7.0-15.0% acetonitrile in 3  
30 min, followed by 15.0-19.0% acetonitrile in 12 min in 100 mM TEAA at a flow rate of  $2.2$   
31  $\mu\text{L}/\text{min}$  (FIG. 6). Again, the chromatogram of the mixture depicted in FIG. 6 with  
32 fragments ranging from 51-587 bp as well as the resolution values given in Table 2  
33 demonstrate that the separation performance of monolithic columns is superior to that of

packed-bed columns with respect to their separation capability for nucleic acids (compare also Figure 1 in Huber et al. *Anal. Chem.* 67:578-585 (1995)).

The separation shown in FIG. 5 was performed under the following conditions:

Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 100 mM TEAA, pH 7.00, buffer B 100 mM TEAA, pH 7.00, 20% acetonitrile; linear gradient, 37-67% B in 3.0 min, 67-87 % B in 7.0 min; flow-rate, 3.1 µL/min; temperature, 50°C; detection, UV, 254 nm; sample, pBR322 DNA-*Hae* III digest, 12.1 ng, 4.5 fmol of each fragment.

The separation shown in FIG. 6 was performed under the following conditions:

Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 100 mM TEAA, pH 7.00, buffer B 100 mM TEAA, pH 7.00, 20% acetonitrile; linear gradient, 35-75% B in 3.0 min, 75-95 % B in 12.0 min; flow-rate, 2.2 µL/min; temperature, 50°C; detection, UV, 254 nm; sample, pBR322 DNA-*Hae* III digest, 1.81 fmol of each fragment.

#### Example 10

##### *Electrospray ionization mass spectrometry and coupling with capillary liquid chromatography*

ESI-MS was performed on a Finnigan MAT LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA, used in FIGs. 10-17) or a Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (used in FIGs. 5 and 6) equipped with an electrospray ion source. The capillary column was directly connected to the spray capillary (fused silica, 105 µm OD, 40 µm ID, Polymicro Technologies) by means of a microtight union (Upchurch Scientific, Oak Harbor, WA, USA). A syringe pump equipped with a 250 µL glass syringe (Unimetrics, Shorewood, IL, USA) was used for continuous infusion experiments and for pumping sheath liquid. For analysis with pneumatically assisted ESI, an electrospray voltage of 3.2-3.7 kV and a nitrogen sheath gas flow of 20-30 arbitrary units (LCQ) or 28-33 psi (TSQ) were employed. The temperature of the heated capillary was set to 200°C. Total ion chromatograms and mass spectra were recorded on a personal computer with the LCQ Navigator software version 1.2 or on a DEC-Alpha 3000 workstation with the ICIS software version 8.3.0 (Finnigan). Mass calibration and coarse tuning was performed in the positive ion mode by direct infusion of a solution of caffeine (Sigma, St. Louis, MO, USA), methionyl-arginyl-phenylalanyl-alanine (Finnigan), and Ultramark 1621 (Finnigan). Fine tuning for ESI-MS of oligodeoxynucleotides in the negative ion mode was performed by infusion of 3.0 µL/min of a 20 pmol/µL solution of (dT)<sub>24</sub> in 25 mM aqueous TEAB containing 10% acetonitrile (v/v). A sheath flow of 3.0 µL/min acetonitrile was added through the triaxial electrospray probe. For all direct infusion experiments, cations present in the

1 oligodeoxynucleotide samples were removed by on-line cation-exchange using a 20 ×  
2 0.50 mm ID cation-exchange microcolumn packed with 38-75 µm Dowex 50 WX8  
3 particles (Serva, Heidelberg, Germany) (Huber et al. M. R. *Anal. Chem.* 70:5288-5295  
4 (1998)). For IP-RP-HPLC-ESI-MS analysis, oligodeoxynucleotides and DNA fragments  
5 were injected without prior cation removal.

#### 6 Example 11

##### 7 *On-line separation and mass determination of synthetic oligodeoxynucleotides*

8 For many of the analytical problems encountered with oligodeoxynucleotides,  
9 chromatographic separation in combination with UV detection is not sufficient to get a  
10 conclusive answer. The on-line conjugation of chromatographic separation to mass  
11 spectrometry, however, offers a potent tool for the characterization and identification of  
12 oligodeoxynucleotides on the basis of accurate mass determinations and fragmentation  
13 patterns. For example, the HPLC-UV analysis of a (dT)<sub>12-18</sub> standard that was left  
14 overnight at room temperature showed a number of small peaks eluting before the  
15 seven major peaks (chromatogram not shown). Applicants supposed that the small  
16 peaks were phosphorylated or non-phosphorylated hydrolysis products of (dT)<sub>12-18</sub>, but  
17 this assumption was not definitive until the separation system was on-line coupled to  
18 ESI-MS, which revealed that they were non-phosphorylated hydrolyzates ranging from  
19 the 6-mer to the 11-mer (FIG. 10). Application of a gradient from 4.0-12.0% acetonitrile  
20 in 10 mM TEAA enabled the separation of all oligothymidylic acids from the 6-mer to the  
21 18-mer. Acetonitrile was added post-column as sheath liquid to enhance the mass  
22 spectrometric detectability of the separated oligodeoxynucleotides (Huber et al. *J.*  
23 *Chromatogr. A* 870:413-424 (2000)). This example demonstrates that by using on-line  
24 IP-RP-HPLC-ESI-MS, the unequivocal identification of low femtomol amounts of  
25 oligodeoxynucleotides is feasible on the basis of their molecular masses (Table 3).

**Table 3****Measured and Theoretical Masses of (dT)<sub>6-18</sub>**

| oligodeoxynucleotide | retention time<br>(min) | molecular mass |             | relative deviation<br>(%) |
|----------------------|-------------------------|----------------|-------------|---------------------------|
|                      |                         | measured       | theoretical |                           |
| (dT) <sub>6</sub>    | 1.77                    | 1763.09        | 1763.21     | 0.006                     |
| (dT) <sub>7</sub>    | 2.63                    | 2066.96        | 2067.40     | 0.021                     |
| (dT) <sub>8</sub>    | 3.59                    | 2371.90        | 2371.59     | -0.013                    |
| (dT) <sub>9</sub>    | 4.35                    | 2675.28        | 2675.79     | 0.019                     |
| (dT) <sub>10</sub>   | 4.94                    | 2978.95        | 2979.98     | 0.035                     |
| (dT) <sub>11</sub>   | 5.44                    | 3284.43        | 3284.18     | -0.008                    |
| (dT) <sub>12</sub>   | 5.76                    | 3589.29        | 3588.37     | -0.026                    |
| (dT) <sub>13</sub>   | 6.13                    | 3892.78        | 3892.57     | -0.006                    |
| (dT) <sub>14</sub>   | 6.39                    | 4197.47        | 4196.76     | -0.017                    |
| (dT) <sub>15</sub>   | 6.66                    | 4501.81        | 4500.96     | -0.019                    |
| (dT) <sub>16</sub>   | 6.92                    | 4806.26        | 4805.15     | -0.023                    |
| (dT) <sub>17</sub>   | 7.12                    | 5109.19        | 5109.35     | 0.003                     |
| (dT) <sub>18</sub>   | 7.35                    | 5413.35        | 5413.54     | 0.004                     |

The separation shown in FIG. 10 was performed under the following conditions:

Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 10 mM TEAA, pH 7.00, buffer B 10 mM TEAA, pH 7.00, 20% acetonitrile; linear gradient, 20-60% B in 10.0 min; flow-rate, 3.2 µL/min; temperature, 50 °C; scan, 800-2000 amu in 2 s; electrospray voltage, 3.8 kV; sheath gas, 34 psi N<sub>2</sub>; sheath liquid, acetonitrile; flow rate, 3.0 µL/min; sample, (dT)<sub>6-18</sub>, 50 ng.

**Example 12***On-line coupling of chromatographic separation to mass spectrometry*

Refined chemistry has significantly improved the efficiency of automated solid-phase synthesis of long oligodeoxynucleotide sequences. However, assuming a coupling efficiency of 98-99% per synthesis cycle, the maximum yield of an 80-mer oligodeoxynucleotide will be only 20-45%, and contamination of the target sequence with a number of failure sequences or partially deprotected sequences is generally observed (Huber et al. *Anal. Chem.* 71:3730-3739 (1999); Huber et al. *LC GC Int.* 14:114-127 (1996)).<sup>28,40</sup> FIG. 11 illustrates the analysis of 5.0 pmol of a crude 80-mer oligodeoxynucleotide. The high number of partly resolved peaks eluting between 2 and

1 6 min made identification and quantitation of the target sequence from the reconstructed  
2 ion chromatogram impossible. However, extraction of a selected ion chromatogram at  
3  $m/z$  1167.0, 1225.5, and 1290.0 clearly identified the target sequence eluting at 3.8 min  
4 (FIG. 12). Averaging and deconvolution of four mass spectra between 3.7 and 3.8 min  
5 yielded a molecular mass of 24,525.0 (FIG. 13 which correlates well with a theoretical  
6 mass of 24,527.17 (0.009% relative deviation). Moreover, the deconvoluted mass  
7 spectrum (FIG. 13) did not show notable cation adduction which verifies that IP-RP-  
8 HPLC is an efficient method for the desalting of oligodeoxynucleotides. Comparison of  
9 the mass spectrum extracted from the chromatogram (FIG. 13) to that of an 80-mer  
10 obtained by direct infusion ESI-MS (compare Figure 3 in Huber et al. *Anal. Chem.*  
11 70:5288-5295 (1998)) clearly corroborates the high value of on-line coupling of  
12 chromatographic separation to mass spectrometry, because the chemical background  
13 in the mass spectrum is greatly reduced upon chromatographic separation and exact  
14 mass measurement is possible using IP-RP-HPLC-ESI-MS with only one fiftieth of the  
15 amount of sample that is consumed during direct infusion ESI-MS.

16 The separations shown in FIGs. 11-13 were performed under the following  
17 conditions: Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 25  
18 mM TEAB, pH 8.40, buffer B 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient,  
19 20-100% B in 15 min; flow-rate, 3.0  $\mu\text{L}/\text{min}$ ; temperature, 50 °C; scan, 1000-3000 amu;  
20 electrospray voltage, 3.2 kV; sheath gas, 30 units; sheath liquid, acetonitrile; flow rate,  
21 3.0  $\mu\text{L}/\text{min}$ ; sample, 5.0 pmol raw product.

## 22 Example 13

### 23 *On-line separation and mass determination of dsDNA fragments*

24 FIG. 14 illustrates the chromatogram of DNA fragments from 486 ng (180 fmol) of  
25 a pBR322 DNA-*Hae* III restriction digest with detection by ESI-MS. For this separation,  
26 the gradient was ramped from 3.0-6.0% acetonitrile in 3.0 min, followed by 6.0-10.0%  
27 acetonitrile in 12 min at a flow rate of 2.8  $\mu\text{L}/\text{min}$  and a column temperature of 40°C.  
28 The elution conditions for the spectra shown in FIGs. 14-17 were as follows: Column,  
29 continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 25 mM TEAB, pH 8.40,  
30 buffer B 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient, 15-30% B in 3.0 min,  
31 followed by 30-50% B in 12 min; flow-rate, 2.8  $\mu\text{L}/\text{min}$ ; temperature, 40°C; scan, 1000-  
32 3000 amu; electrospray voltage, 3.2 kV; sheath gas, 32 units; sheath liquid, acetonitrile;  
33 flow rate, 3  $\mu\text{L}/\text{min}$ ; sample, pBR322 DNA-*Hae* III digest, 180 fmol of each fragment.

1           It can be seen that the fragments from 51-123 bp were completely resolved in the  
2 chromatogram, whereas the separation of the longer fragments was incomplete due to  
3 overloading of the column (Oberacher, H.; Krajete, A.; Parson, W.; Huber, C. G. J.  
4 *Chromatogr. A submitted (2000)*). Mass spectra were extracted from the reconstructed  
5 ion chromatogram by averaging 4-8 scans and three examples for fragments ranging in  
6 size from 80 to 267 bp are illustrated in FIGs. 15-17. Whereas relatively few charge  
7 states (23- - 29-) were found in the mass spectrum of an 80 bp fragment (FIG. 15), the  
8 number of observed signals rapidly increased with the size of the DNA fragments (FIGs.  
9 16 and 17). The appearance of all charge state signals with sharp and defined peak  
10 shapes indicates, that cation adducts have been efficiently removed by IP-RP-HPLC.

11           The molecular mass of the DNA fragments was calculated by a three step  
12 procedure. First, a rough molecular mass was obtained by automatic deconvolution of  
13 the raw spectrum using the Bioworks software application. For the fragments from 51-  
14 267 bp this deconvolution step readily yielded definite mass information and even the  
15 mass spectrum of the coeluting 123 bp and 124 bp fragments was easily deconvoluted  
16 into two separate mass peaks. For the longer DNA fragments (434-587 bp), signals for  
17 the individual charge states could be only identified using the knowledge of the  
18 theoretical molecular mass of the investigated fragments from their DNA sequence.  
19 Subsequently, the charge states of all  $m/z$  signals in the mass spectrum having an  
20 abundance more than five times the signal-to-noise ratio were calculated. Finally, the  
21  $m/z$  values and the corresponding integer charges state were used to calculate a  
22 molecular mass. Statistical treatment of the molecular masses of the individual charge  
23 states gave the average molecular mass and its standard deviation. The results of these  
24 calculations are summarized in (Table 4), which shows that the masses of the double-  
25 stranded DNA fragments ranging in size up to 267 bp were measured with an accuracy  
26 of better than 0.08%.

Table 4

**Molecular Masses of Double-stranded DNA Fragments from the pBR322 DNA-*Hae* III Digest**

| fragment | position <sup>a)</sup> | molecular mass         |             | relative deviation (%) |
|----------|------------------------|------------------------|-------------|------------------------|
|          |                        | measured <sup>b)</sup> | theoretical |                        |
| 51       | 942-992                | 31,565±24 (4)          | 31,559.57   | 0.018                  |
| 57       | 993-1049               | 35,252±54 (6)          | 35,263.04   | -0.032                 |
| 64       | 534-597                | 39,573±84 (7)          | 39,592.83   | -0.026                 |
| 80       | 3410-3489              | 49,494±43 (10)         | 49,475.35   | 0.038                  |
| 89       | 832-920                | 55,058±41 (14)         | 55,038.97   | 0.034                  |
| 104      | 298-401                | 64,391±56 (22)         | 64,312.99   | 0.12                   |
| 123      | 175-297                | 76,059±49 (15)         | 76,045.76   | 0.017                  |
| 124      | 402-525                | 76,731±44 (17)         | 76,675.05   | 0.073                  |
| 184      | 1263-1446              | 113,802±140 (15)       | 113,747.36  | 0.048                  |
| 192      | 4344-174               | 118,722±123 (17)       | 118,668.82  | 0.045                  |
| 213      | 1050-1262              | 131,733±148 (18)       | 131,674.02  | 0.045                  |
| 234      | 598-831                | 144,708±127 (25)       | 144,646.56  | 0.042                  |
| 267      | 3490-3756              | 165,091±230 (12)       | 165,019.11  | 0.044                  |
| 434      | 2518-2951              | n. d. <sup>c)</sup>    | 268,240.41  | n. d.                  |
| 458      | 2952-3409              | n. d.                  | 283,002.81  | n. d.                  |
| 502      | 1447-1948              | n. d.                  | 310,240.12  | n. d.                  |
| 540      | 1949-2488              | n. d.                  | 333,738.33  | n. d.                  |
| 587      | 3757-4343              | n. d.                  | 362,707.09  | n. d.                  |

<sup>a)</sup>position relative to the *EcoR* I restriction site in pBR322.

<sup>b)</sup>molecular mass given as average±standard deviation (number of charge states used to calculate the average molecular mass).

<sup>c)</sup>not determined.

**Example 14**

***IP-RP-HPLC-ESI-MS/MS sequencing of oligodeoxynucleotides***

In addition to information regarding the molecular mass, tandem mass spectrometry (MS/MS) utilizing collisionally induced dissociation (CID) provides valuable

1 information about the base sequence of oligodeoxynucleotides (McLuckey et al.  
2 *Tandem Mass Spectrometry of Small, Multiply Charged Oligodeoxynucleotides* 3:pp 60-  
3 70 (1992); Griffey et al. *J. Mass Spectrom.* 32:305-313 (1997)). In this example, for the  
4 application of monolithic capillary columns in nucleic acid analysis, the feasibility to  
5 perform on-line MS/MS experiments on oligodeoxynucleotides upon liquid  
6 chromatographic separation was examined. To evaluate the performance of IP-RP-  
7 HPLC-ESI-MS/MS for oligodeoxynucleotide sequencing, a 5-mer oligodeoxynucleotide  
8 (sequence 5'-ATGCG-3') was ordered from Microsynth. The IP-RP-HPLC-ESI-MS  
9 analysis of the unfragmented 5-mer gave a molecular mass of 1805.00, which  
10 exceeded the expected mass value of 1503.04 by 301.96 mass units. This mass  
11 difference could be attributable to an additional thymidine residue (probably entered into  
12 the synthesis automat by accident) or to a 5'-terminal dimethoxytrityl protecting group  
13 (that has been forgotten to hydrolyze after the last coupling cycle). Substantially  
14 increased retention in the chromatographic analysis was indicative for the latter  
15 assumption. The presence of a dimethoxytrityl protecting group as well as the total  
16 sequence of the oligodeoxynucleotide was confirmed using IP-RP-HPLC-ESI-MS/MS  
17 (FIGs. 18 and 19). The ESI-MS/MS experiment was performed by isolating the  $[M-2H]^{2-}$   
18 charge state at  $m/z$  901.37 and collisional activation at 19% relative collision energy.  
19 Assignments and masses for the fragment ions observed in the tandem mass spectrum  
20 (FIG .19) are listed in Table 5.  
21

Table 5

## Fragment Ions for Sequencing of a 5-mer Oligodeoxynucleotide

| Ion assignment                    | m/z     |
|-----------------------------------|---------|
| (M) <sup>2-</sup>                 | 901.37  |
| (M-A) <sup>2-</sup>               | 833.65  |
| (M-T) <sup>2-</sup>               | 838.57  |
| (M-G) <sup>2-</sup>               | 826.13  |
| (M-C) <sup>2-</sup>               | 845.89  |
| (w <sub>1</sub> ) <sup>1-</sup>   | 345.87  |
| (w <sub>2</sub> ) <sup>1-</sup>   | 635.06  |
| (w <sub>3</sub> ) <sup>1-</sup>   | 964.18  |
| (w <sub>4</sub> ) <sup>1-</sup>   | 1267.01 |
| (w <sub>3</sub> ) <sup>2-</sup>   | 481.45  |
| (w <sub>4</sub> ) <sup>2-</sup>   | 633.53  |
| (a <sub>2</sub> -T) <sup>1-</sup> | 714.16  |
| (a <sub>3</sub> -G) <sup>1-</sup> | 1016.07 |
| (a <sub>4</sub> -C) <sup>1-</sup> | 1345.98 |

Beside the parent ion all four ions that show loss of one nucleobase are observed. The most diagnostic ions however arise from fragmentation which produces w series ions, that are used to determine the 3'→5' sequence and the a<sub>n</sub>-B<sub>n</sub> series ions, that are used to determine the 5'→3' sequence (McLucky et al. *Tandem Mass Spectrometry of Small, Multiply Charged Oligodeoxynucleotides* 3:60-70 (1992)). The complete w series is present in the MS/MS spectrum and the masses correspond to those expected for an oligodeoxynucleotide with the sequence 5'-ATGCG-3', proving that the 3' terminus is unmodified. The a<sub>n</sub>-B<sub>n</sub> series however shows a mass shift of +302 from the expected mass, corresponding to the presence of the dimethoxytrityl protecting group at the 5' terminus. Finally the presence of the protective group was confirmed by cleavage with 2% formic acid at room temperature for 5 minutes, yielding the oligodeoxynucleotide ATGCG with the expected mass of 1502.98.

The separations shown in FIGs. 18 and 19 were performed under the following conditions: Column, continuous PS-DVB, 60 × 0.20 mm ID; mobile phase, buffer A 25 mM TEAB, pH 8.40, buffer B 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient,

1 10-100% B in 5.0 min; flow-rate, 3.0  $\mu$ L/min; temperature, 50 °C; daughter ions of m/z  
2 901.5, 4.0 amu isolation width, 19% relative collision energy; scan, 250-1810 amu;  
3 electrospray voltage, 3.2 kV; sheath gas, 30 units; sheath liquid, acetonitrile; flow rate,  
4 3.0  $\mu$ L/min; sample, 25 pmol raw product.

5

6 While the foregoing has presented specific embodiments of the present  
7 invention, it is to be understood that these embodiments have been presented by way of  
8 example only. It is expected that others will perceive and practice variations which,  
9 though differing from the foregoing, do not depart from the spirit and scope of the  
10 invention as described and claimed herein.

1 The invention claimed is:

- 2 1. A method for separating a mixture of polynucleotides, said method comprising:  
3 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
4 chromatographic surfaces and eluting said mixture of polynucleotides with a  
5 mobile phase comprising a counterion agent and an organic solvent,  
6 wherein said monolith is contained within a fused silica tube having an inner  
7 diameter in the range of 1 micrometer to 1000 micrometer,  
8 wherein said monolith is immobilized by covalent attachment at the inner wall of  
9 said tube, and  
10 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
11 matrix.
- 12 2. A method of claim 1 wherein said tube is devoid of retaining frits.
- 13 3. A method of claim 1 wherein said monolith is characterized by having 100,000 to  
14 200,000 theoretical plates per meter.
- 15 4. A method of claim 3 wherein said theoretical plates per meter is determined from the  
16 retention time of single stranded p(dT)<sub>18</sub> standard using the following equation:  
17

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

- 18 wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
19 standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half  
20 height, and  $L$  is the length of the monolith in meters.
- 21 5. A method of claim 4 wherein said tube has an inner diameter of 200 micrometer and  
22 a length of 60 mm, wherein during said isocratic elution said monolith has a back  
23 pressure in the range of 180 to 200 bar, and a flow rate in the range of 2 to 3  $\mu\text{L}/$   
24 min at an elution temperature of 50°C.
- 25 6. A method of claim 1 wherein said mobile phase is devoid of EDTA.
- 26 7. A method of claim 1 wherein said monolith has a surface morphology, as determined  
27 by scanning electron microscopy, that resembles the surface morphology of  
28 octadecyl modified poly(styrene-divinylbenzene) particles, wherein said surface  
29 morphology of said monolith is brush-like.
- 30 8. A method of claim 1 wherein said monolith has a surface morphology, as determined  
31 by scanning electron microscopy, that resembles the surface morphology of

- 1 octadecyl modified poly(styrene-divinylbenzene) particles, wherein said surface  
2 morphology of said monolith is rugulose.  
3
- 4 9. A method for separating a mixture of polynucleotides, said method comprising:  
5 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
6 chromatographic surfaces and eluting said mixture of polynucleotides with a  
7 mobile phase comprising a counterion agent and an organic solvent,  
8 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
9 matrix,  
10 wherein said monolith is contained within a fused silica tube, and  
11 wherein said monolith is immobilized by covalent attachment at the inner wall of  
12 said tube.
- 13 10. A method of claim 9 wherein said monolith is contained within said fused silica tube  
14 having an inner diameter in the range of 1 micrometer to 1000 micrometer.
- 15 11. A method of claim 9 wherein said tube is devoid of retaining frits.
- 16 12. A method of claim 9 wherein said monolith is characterized by having 100,000 to  
17 200,000 theoretical plates per meter.
- 18 13. A method of claim 9 wherein said mobile phase is devoid of EDTA.
- 19 14. A method of claim 9 wherein said monolith has a surface morphology, as  
20 determined by scanning electron microscopy, that resembles the surface  
21 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
22 said surface morphology of said monolith is brush-like.
- 23 15. A method of claim 9 wherein said monolith has a surface morphology, as  
24 determined by scanning electron microscopy, that resembles the surface  
25 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
26 said surface morphology of said monolith is rugulose.  
27
- 28 16. A method for separating a mixture of polynucleotides, said method comprising:  
29 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
30 chromatographic surfaces and eluting said mixture of polynucleotides with a  
31 mobile phase comprising a counterion agent and an organic solvent,  
32 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
33 matrix,  
34 wherein said monolith is contained within a fused silica tube,

- 1        wherein said tube has an inner diameter in the range of 1 micrometer to 1000  
2        micrometer,  
3        wherein said tube is devoid of retaining frits, and  
4        wherein said polynucleotides comprise double-stranded fragments having  
5        lengths in the range of 3 to 600 base pairs.
- 6    17. A method of claim 16 wherein said mobile phase is devoid of EDTA.
- 7    18. A method of claim 17 wherein said monolith is immobilized by covalent attachment  
8        at the inner wall of said tube.
- 9    19. A method of claim 16 wherein said monolith is characterized by having 100,000 to  
10       200,000 theoretical plates per meter.
- 11   20. A method of claim 16 wherein said monolith has a surface morphology, as  
12       determined by scanning electron microscopy, that resembles the surface  
13       morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
14       said surface morphology of said monolith is brush-like.
- 15   21. A method of claim 16 wherein said monolith is characterized by having at least  
16       100,000 theoretical plates per meter.
- 17   22. A method of claim 16 wherein said monolith has a surface morphology, as  
18       determined by scanning electron microscopy, that resembles the surface  
19       morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
20       said surface morphology of said monolith is rugulose.
- 21   23. A method for separating a mixture of polynucleotides, said method comprising:  
22       applying said mixture of polynucleotides to a polymeric monolith having non-polar  
23       chromatographic surfaces and eluting said mixture of polynucleotides with a  
24       mobile phase comprising a counterion agent and an organic solvent,  
25       wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
26       matrix,  
27       wherein said monolith is characterized by having 10,000 to 200,000 theoretical  
28       plates per meter,  
29       wherein said monolith is contained within a fused silica tube having an inner  
30       diameter in the range of 1 micrometer to 1000 micrometer, and  
31       wherein said monolith is immobilized by covalent attachment at the inner wall of  
32       said tube.
- 33   24. A method of claim 23 wherein said theoretical plates per meter is determined from  
34       the retention time of single stranded p(dT)<sub>18</sub> standard using the following  
35       equation:

1

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

2        wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
3        standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half  
4        height, and  $L$  is the length of the monolith in meters.

5        25. A method of claim 23 wherein said monolith has a surface morphology, as  
6        determined by scanning electron microscopy, that resembles the surface  
7        morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
8        said surface morphology of said monolith is brush-like

9        26. A method of claim 23 wherein said tube is silanized.

10       27. A method of claim 23 wherein said tube is devoid of retaining frits.

11       28. A method of claim 23 wherein said mobile phase is devoid of EDTA.

12       29. A method of claim 23 wherein said monolith has a surface morphology, as  
13       determined by scanning electron microscopy, that resembles the surface  
14       morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
15       said surface morphology of said monolith is rugulose.

16       30. A method for separating a mixture of polynucleotides, said method comprising:  
17       applying said mixture of polynucleotides to a polymeric monolith having non-polar  
18       chromatographic surfaces and eluting said mixture of polynucleotides with a  
19       mobile phase comprising a counterion agent and an organic solvent,  
20       wherein said monolith is contained within a fused silica tube having an inner  
21       diameter in the range of 1 micrometer to 1000 micrometer,  
22       wherein said mobile phase is devoid of EDTA,  
23       wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
24       matrix

25       31. A method of claim 30 wherein said monolith has a surface morphology, as  
26       determined by scanning electron microscopy, that resembles the surface  
27       morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
28       said surface morphology of said monolith is brush-like.

29       32. A method of claim 30 wherein said monolith is immobilized by covalent attachment  
30       at the inner wall of said tube.

31       33. A method of claim 32 wherein said tube is devoid of retaining frits.

- 1 34. A method of claim 30 wherein said monolith is characterized by having 10,000 to  
2 200,000 theoretical plates per meter.
- 3 35. A method of claim 30 wherein said monolith has a surface morphology, as  
4 determined by scanning electron microscopy, that resembles the surface  
5 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
6 said surface morphology of said monolith is rugulose.
- 7 36. A method of claim 30 wherein said tube has been silanized.  
8
- 9 37. A method for separating a mixture of polynucleotides, said method comprising:  
10 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
11 chromatographic surfaces and eluting said mixture of polynucleotides with a  
12 mobile phase comprising a counterion agent and an organic solvent,  
13 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
14 matrix,  
15 wherein said monolith has a surface morphology, as determined by scanning  
16 electron microscopy, that resembles the surface morphology of octadecyl  
17 modified poly(styrene-divinylbenzene) particles, wherein said surface  
18 morphology of said monolith is rugulose.
- 19 38. A method of claim 37 wherein said mobile phase is devoid of EDTA.
- 20 39. A method of claim 37 wherein said monolith is contained within a fused silica tube  
21 having an inner diameter in the range of 1 micrometer to 1000 micrometer.
- 22 40. A method of claim 37 wherein said monolith is immobilized by covalent attachment  
23 at the inner wall of said tube.
- 24 41. A method of claim 37 wherein said tube is devoid of retaining frits.
- 25 42. A method of claim 37 wherein said monolith is characterized by having 100,000 to  
26 200,000 theoretical plates per meter.
- 27 43. A method of claim 37 wherein said monolith has a surface morphology, as  
28 determined by scanning electron microscopy, that resembles the surface  
29 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
30 said surface morphology of said monolith is brush-like.  
31
- 32 44. A method for separating a mixture of polynucleotides, said method comprising:  
33 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
34 chromatographic surfaces and eluting said mixture of polynucleotides with a  
35 mobile phase comprising a counterion agent and an organic solvent,

- 1        wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
2        matrix,  
3        wherein said monolith is contained within a fused silica tube having an inner  
4        diameter in the range of 1 micrometer to 1000 micrometer,  
5        wherein said monolith is immobilized at the inner wall of said tube,  
6        wherein said tube is devoid of retaining frits.
- 7    45. A method of claim 44 wherein said mobile phase is devoid of EDTA.
- 8    46. A method of claim 44 wherein said monolith is contained within a tube having an  
9        inner diameter in the range of 10 micrometer to 300 micrometer.
- 10   47. A method of claim 44 wherein said monolith is immobilized at the inner wall of said  
11        tube and wherein said tube has been silanized.
- 12   48. A method of claim 44 wherein said monolith has a surface morphology, as  
13        determined by scanning electron microscopy, that resembles the surface  
14        morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
15        said surface morphology of said monolith is brush-like.
- 16   49. A method of claim 44 wherein said monolith is characterized by having 100,000 to  
17        200,000 theoretical plates per meter.
- 18   50. A method of claim 44 wherein said monolith has a surface morphology, as  
19        determined by scanning electron microscopy, that resembles the surface  
20        morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
21        said surface morphology of said monolith is rugulose.
- 22
- 23   51. A device for separating a mixture of polynucleotides, said device comprising:  
24        a polymeric monolith having non-polar chromatographic surfaces,  
25        wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
26        matrix,  
27        wherein said monolith is contained within a fused silica tube having an inner  
28        diameter in the range of 1 micrometer to 1000 micrometer, wherein said monolith  
29        is immobilized by covalent attachment at the inner wall of said tube.
- 30   52. A device of claim 51 wherein said tube is devoid of retaining frits.
- 31   53. A device of claim 51 wherein said monolith is characterized by having 100,000 to  
32        200,000 theoretical plates per meter.
- 33   54. A device of claim 53 wherein said theoretical plates per meter is determined from  
34        the retention time of single stranded p(dT)<sub>18</sub> standard using the following  
35        equation:

1

$$(N/L) = (5.54/L) \left( \frac{t_R}{w_{0.5}} \right)^2$$

2        wherein  $N$  is the number of theoretical plates,  $t_R$  is the retention time of said  
3        standard determined during an isocratic elution,  $w_{0.5}$  is the peak width at half  
4        height, and  $L$  is the length of the monolith in meters.

5        55. A device of claim 54 wherein said tube has an inner diameter of 200 micrometer  
6        and a length of 60 mm, wherein during said isocratic elution said monolith has a  
7        back pressure in the range of 180 to 200 bar, and a flow rate in the range of 2 to  
8        3  $\mu\text{L}/\text{min}$  at an elution temperature of  $50^\circ\text{C}$ .

9        56. A device of claim 51 wherein said monolith has a surface morphology, as  
10       determined by scanning electron microscopy, that resembles the surface  
11       morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
12       said surface morphology of said monolith is rugulose.

13       57. A device of claim 51 wherein the chromatographic surfaces of said monolith are  
14       devoid of micropores.

15       58. A device of claim 57 wherein said monolith has channels sufficiently large for  
16       convective flow of said mobile phase.

17

18       59. A device for separating a mixture of polynucleotides, said device comprising:  
19       a polymeric monolith having non-polar chromatographic surfaces,  
20       wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
21       matrix,  
22       wherein said monolith is contained within a fused silica tube, and  
23       wherein said monolith is immobilized by covalent attachment at the inner wall of  
24       said tube.

25       60. A device of claim 59 wherein said tube has an inner diameter in the range of 1  
26       micrometer to 1000 micrometer.

27       61. A device of claim 59 wherein said tube is devoid of retaining frits.

28       62. A device of claim 59 wherein said monolith is characterized by having 10,000 to  
29       200,000 theoretical plates per meter.

30       63. A device of claim 59 wherein said monolith has a surface morphology, as  
31       determined by scanning electron microscopy, that resembles the surface

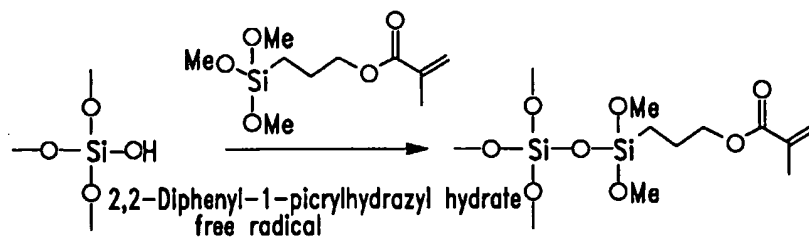
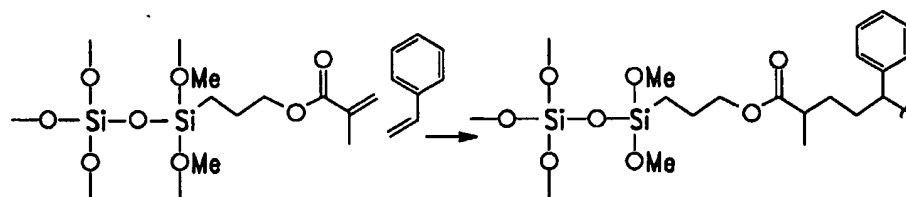
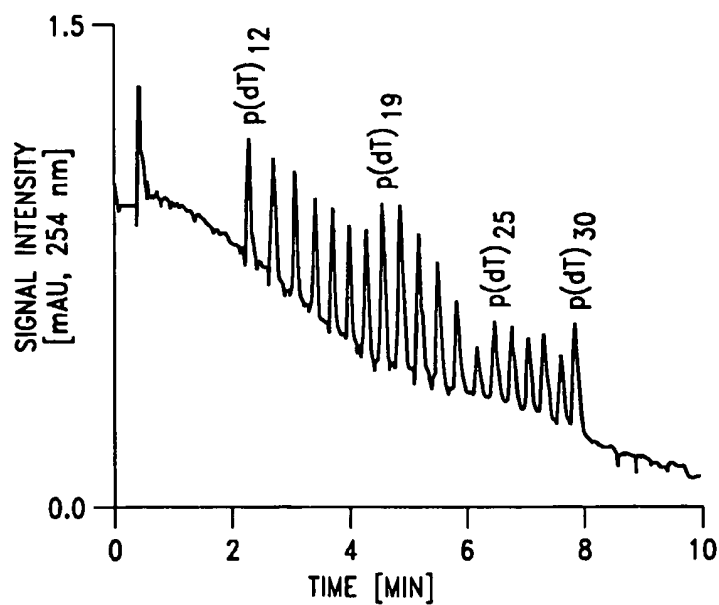
- 1 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
2 said surface morphology of said monolith is brush-like.
- 3 64. A device of claim 59 wherein said monolith comprises an underivatized monolithic  
4 stationary phase.
- 5 65. A device of claim 59 wherein said monolith has a surface morphology, as  
6 determined by scanning electron microscopy, that resembles the surface  
7 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
8 said surface morphology of said monolith is rugulose.
- 9 66. A device of claim 59 wherein said monolith is devoid of micropores and wherein  
10 said monolith has channels sufficiently large for convective flow of said mobile  
11 phase.  
12
- 13 67. A device for separating a mixture of polynucleotides, said device comprising:  
14 a polymeric monolith having non-polar chromatographic surfaces,  
15 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
16 matrix,  
17 wherein said monolith is contained within a fused silica tube,  
18 wherein said tube has been silanized, and  
19 wherein said tube is devoid of retaining frits.
- 20 68. A device of claim 67 wherein said monolith is immobilized by covalent attachment  
21 at the inner wall of said tube.
- 22 69. A device of claim 67 wherein said monolith is characterized by having 100,000 to  
23 200,000 theoretical plates per meter.
- 24 70. A device of claim 67 wherein said monolith has a surface morphology, as  
25 determined by scanning electron microscopy, that resembles the surface  
26 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
27 said surface morphology of said monolith is brush-like.
- 28 71. A device of claim 67 wherein said tube has an inner diameter in the range of 1  
29 micrometer to 1000 micrometer.
- 30 72. A device of claim 67 wherein said monolith has a surface morphology, as  
31 determined by scanning electron microscopy, that resembles the surface  
32 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
33 said surface morphology of said monolith is rugulose.  
34
- 35 73. A device for separating a mixture of polynucleotides, said device comprising:

- 1 a polymeric monolith having non-polar chromatographic surfaces,  
2 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
3 matrix,  
4 wherein said monolith is contained within a tube having an inner diameter in the  
5 range of 1 micrometer to 1000 micrometer,  
6 wherein said monolith is characterized by having 10,000 to 200,000 theoretical  
7 plates per meter.
- 8 74. A device of claim 73 wherein said monolith is contained within a tube having an  
9 inner diameter in the range of 1 micrometer to 1000 micrometer.
- 10 75. A device of claim 73 wherein said monolith is immobilized by covalent attachment  
11 at the inner wall of said tube.
- 12 76. A device of claim 75 wherein said tube is devoid of retaining frits.
- 13 77. A method of claim 73 wherein said monolith has a surface morphology, as  
14 determined by scanning electron microscopy, that resembles the surface  
15 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
16 said surface morphology of said monolith is brush-like.
- 17 78. A method of claim 73 wherein said monolith has a surface morphology, as  
18 determined by scanning electron microscopy, that resembles the surface  
19 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
20 said surface morphology of said monolith is rugulose.
- 21
- 22 79. A device for separating a mixture of polynucleotides, said device comprising:  
23 a polymeric monolith having non-polar chromatographic surfaces,  
24 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
25 matrix,  
26 wherein said monolith is characterized by having at least 100,000 theoretical  
27 plates per meter,  
28 wherein said monolith is contained within a silanized fused silica tube having an  
29 inner diameter in the range of 10 micrometer to 1000 micrometer,  
30 wherein said monolith is immobilized at the inner wall of said tube.
- 31 80. A device of claim 79 wherein said monolith is characterized by having 100,000 to  
32 200,000 theoretical plates per meter.
- 33 81. A device of claim 79 wherein said monolith is contained within a tube having an  
34 inner diameter in the range of 1 micrometer to 1000 micrometer.

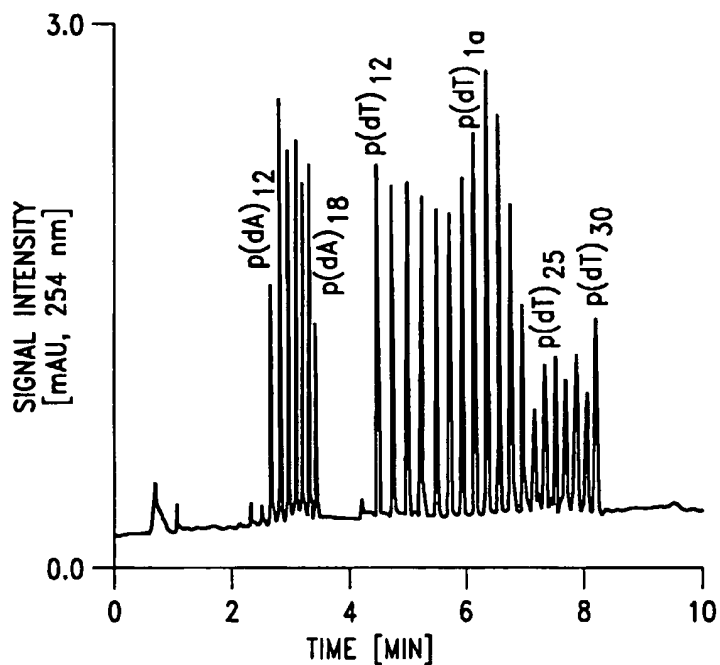
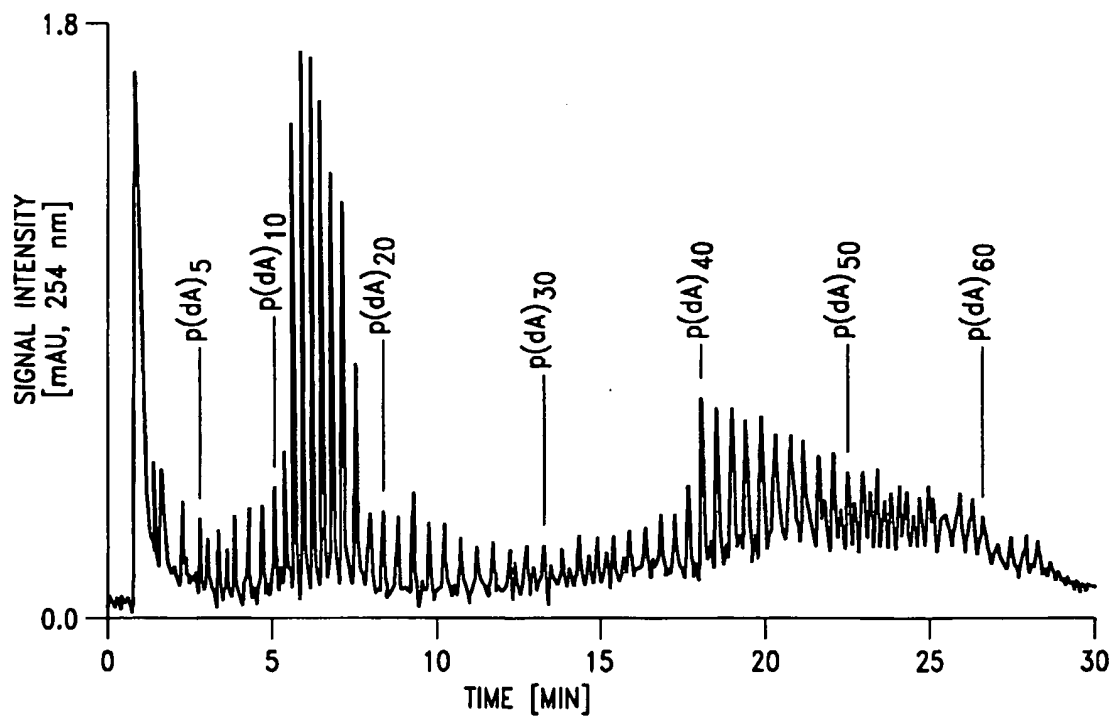
- 1 82. A device of claim 79 wherein said monolith has a surface morphology, as  
2 determined by scanning electron microscopy, that resembles the surface  
3 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
4 said surface morphology of said monolith is brush-like.
- 5 83. A device of claim 82 wherein said tube is devoid of retaining frits.
- 6 84. A device of claim 79 wherein said monolith has a surface morphology, as  
7 determined by scanning electron microscopy, that resembles the surface  
8 morphology of octadecyl modified poly(styrene-divinylbenzene) particles, wherein  
9 said surface morphology of said monolith is rugulose.
- 10 85. A miniaturized chromatographic system for separating a mixture of polynucleotides,  
11 said system comprising the device of claim 79.
- 12 86. A device for separating a mixture of polynucleotides, said device comprising:  
13 a polymeric monolith having non-polar chromatographic surfaces,  
14 wherein said monolith has a surface morphology, as determined by scanning  
15 electron microscopy, that resembles the surface morphology of octadecyl  
16 modified poly(styrene-divinylbenzene) particles, wherein said surface  
17 morphology of said monolith is rugulose and brush-like,  
18 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
19 matrix,  
20 wherein said monolith is contained within a fused silica tube having an inner  
21 diameter in the range of 1 micrometer to 1000 micrometer,  
22 wherein said monolith is immobilized at the inner wall of said tube.
- 23 87. A device of claim 86 wherein said tube is devoid of retaining frits.
- 24 88. A device of claim 86 wherein said monolith is characterized by having 100,000 to  
25 200,000 theoretical plates per meter.
- 26 89. A device of claim 86 wherein said tube has been silanized.
- 27 90. A device of claim 86 wherein said surfaces of said monolith are non-porous.
- 28 91. A device of claim 86 wherein said monolith is formed from a polymerization mixture  
29 including underivatized styrene, a crosslinking agent, and a porogen, wherein  
30 said porogen comprises tetrahydrofuran.
- 31 92. A device of claim 86 wherein said polynucleotides comprise double-stranded  
32 fragments having lengths in the range of 3 to 600 base pairs.
- 33 93. A method of claim 16 including analyzing eluted polynucleotides by mass spectral  
34 analysis.

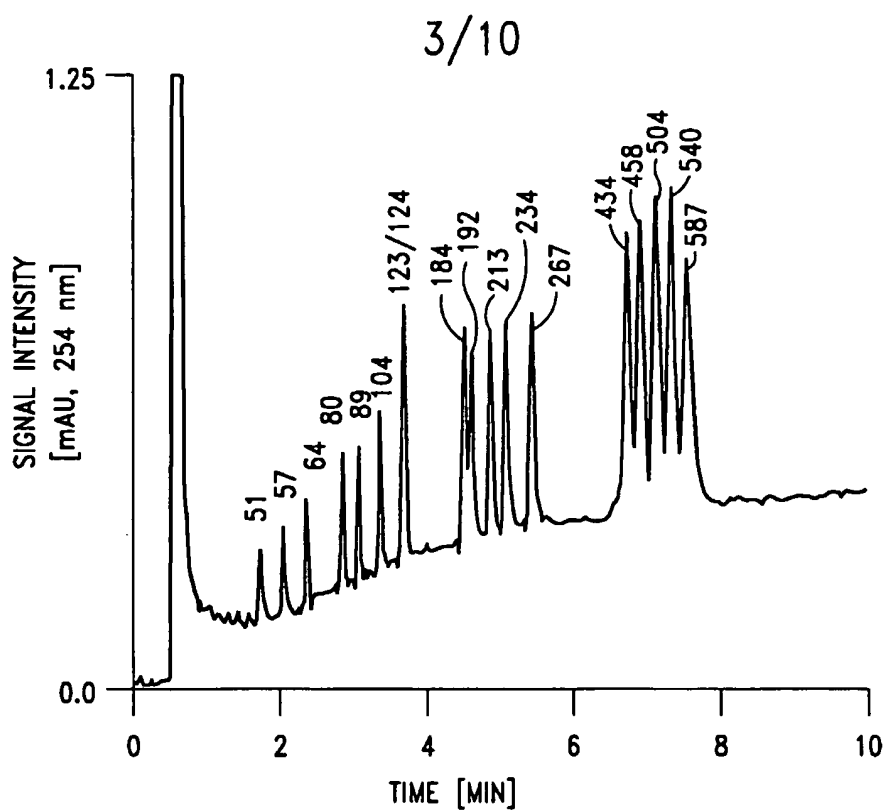
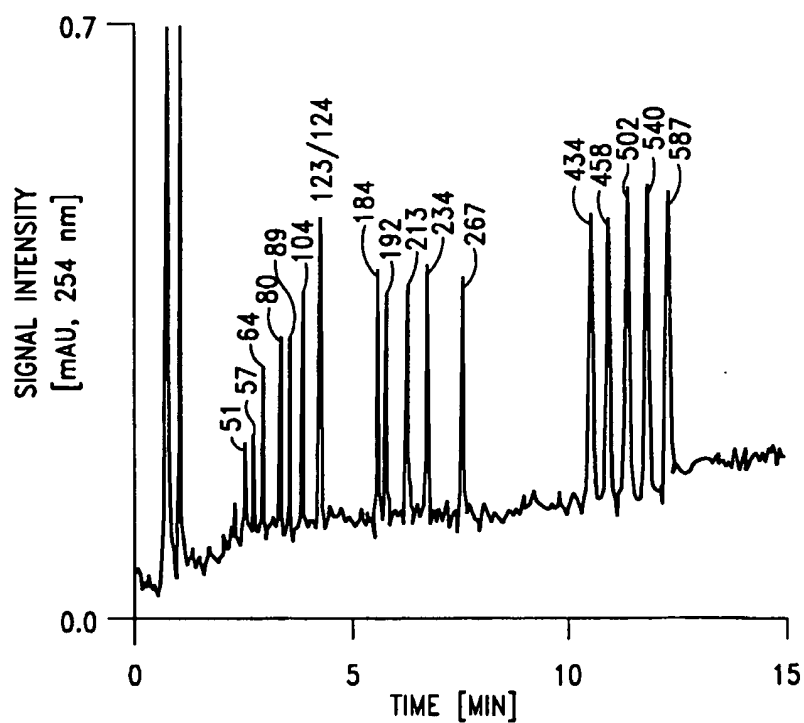
- 1 94. A method of claim 23 including analyzing eluted polynucleotides by mass spectral  
2 analysis.
- 3 95. A system of claim 85 wherein said monolith is operatively coupled to a mass  
4 spectrometer.
- 5 96. A method for desalting a mixture of polynucleotides, said method comprising:  
6 applying said mixture of polynucleotides to a polymeric monolith having non-polar  
7 chromatographic surfaces and eluting said mixture of polynucleotides with a  
8 mobile phase comprising a counterion agent and an organic solvent,  
9 wherein said monolith is characterized by having 100,000 to 200,000 theoretical  
10 plates per meter,  
11 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
12 matrix,  
13 wherein said monolith is contained within a fused silica tube having an inner  
14 diameter in the range of 1 micrometer to 1000 micrometer,  
15 wherein said monolith is immobilized at the inner wall of said tube.
- 16 97. A chromatographic device, said device comprising:  
17 a polymeric monolith having non-polar chromatographic surfaces,  
18 wherein said monolith comprises an underivatized poly(styrene-divinylbenzene)  
19 matrix,  
20 wherein said monolith is contained within a silanized fused silica tube having an  
21 inner diameter in the range of 10 micrometer to 1000 micrometer, and  
22 wherein said monolith is immobilized at the inner wall of said tube.

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*FIG. - 1 a**FIG. - 1 b**FIG. - 2*

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*FIG.-3**FIG.-4*

*FIG.-5**FIG.-6*

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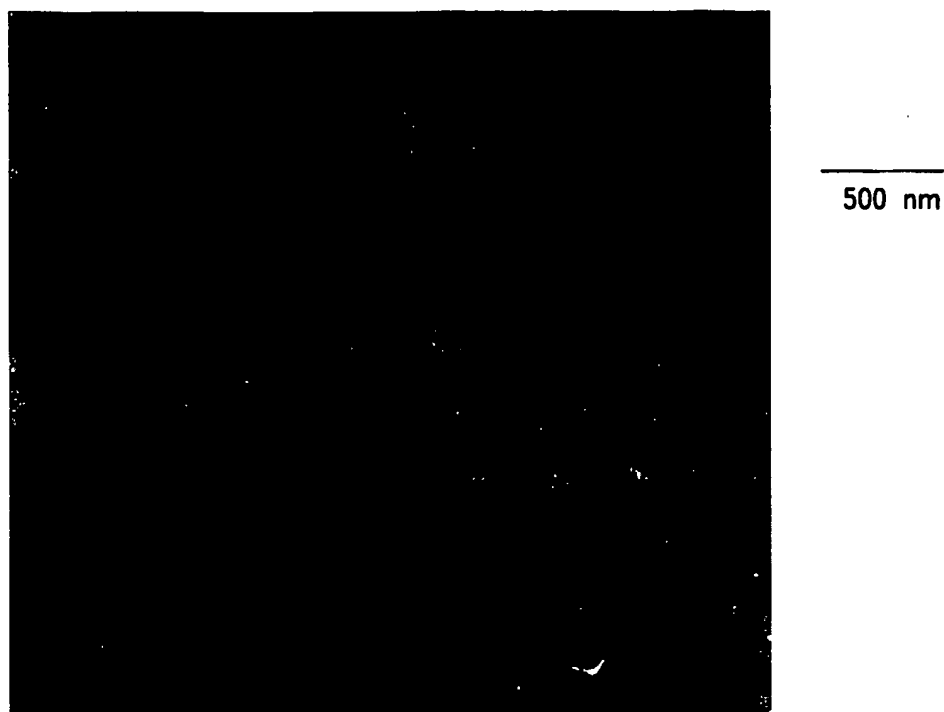
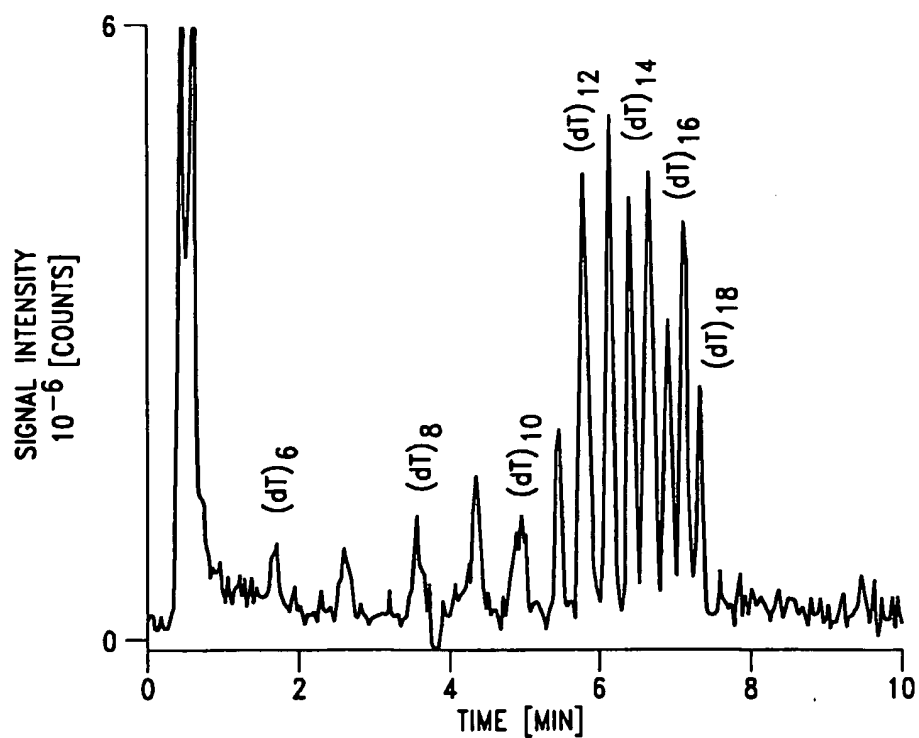


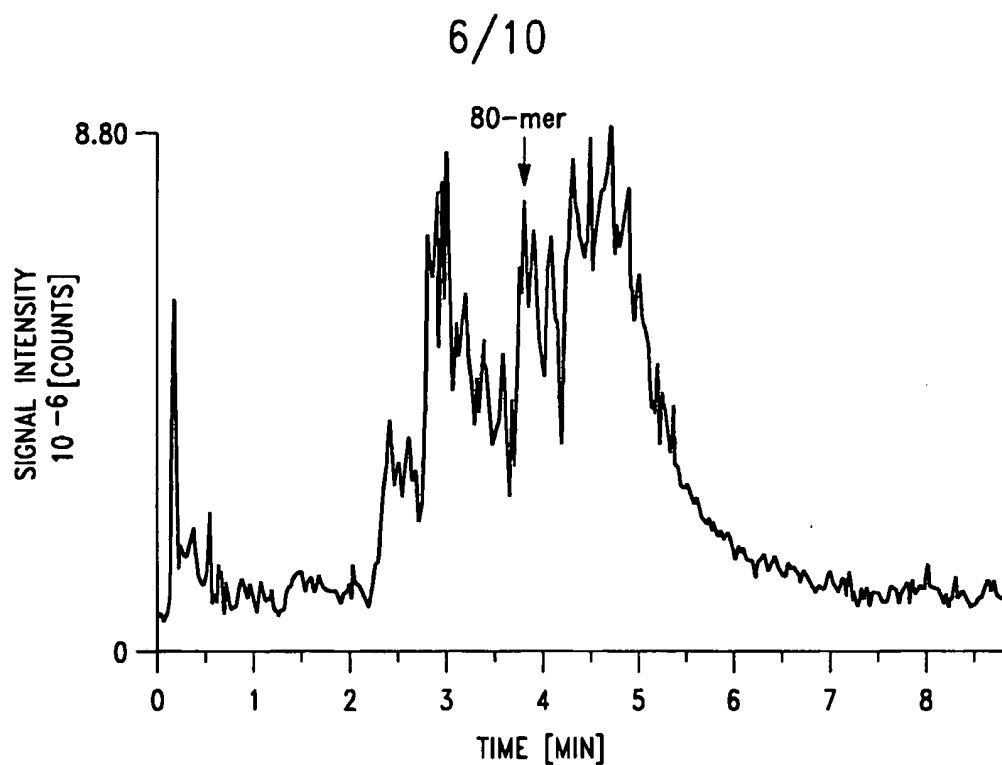
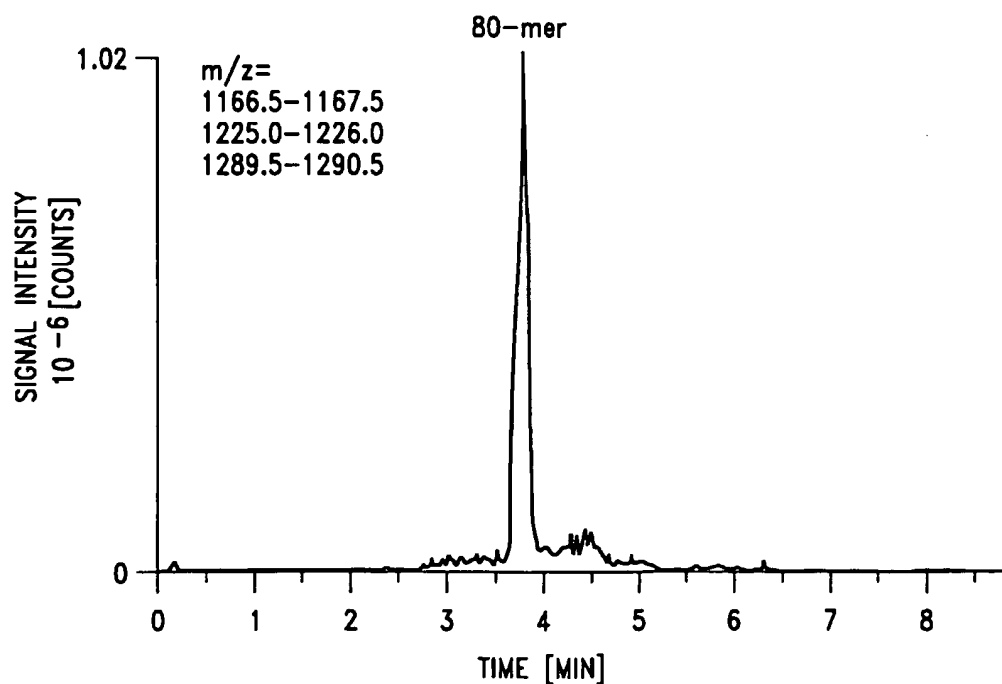
*FIG.-7*



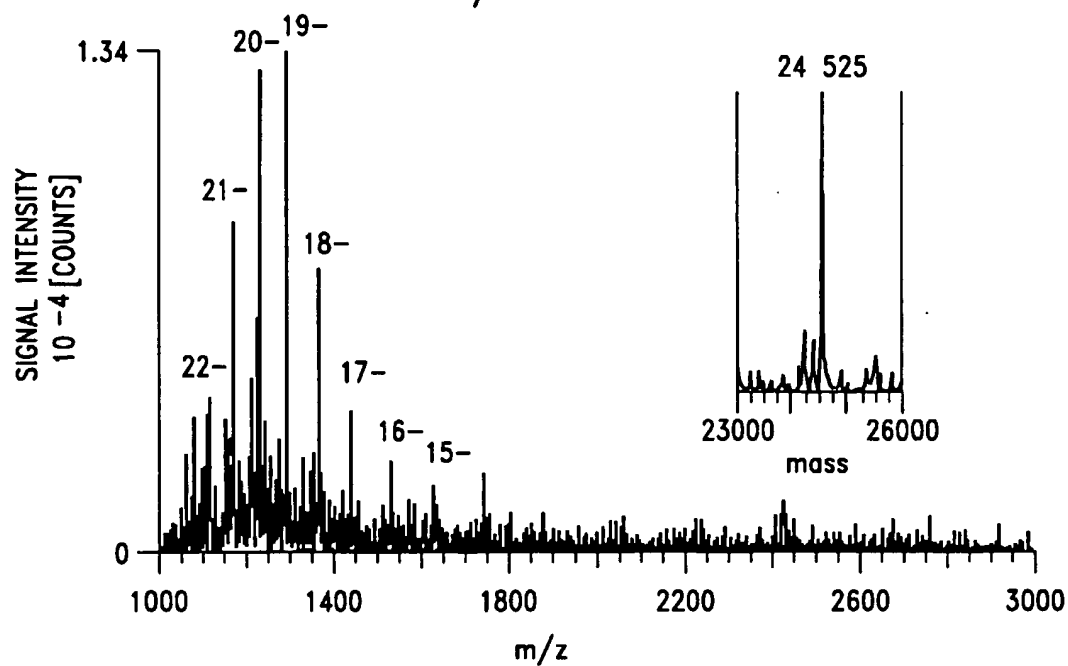
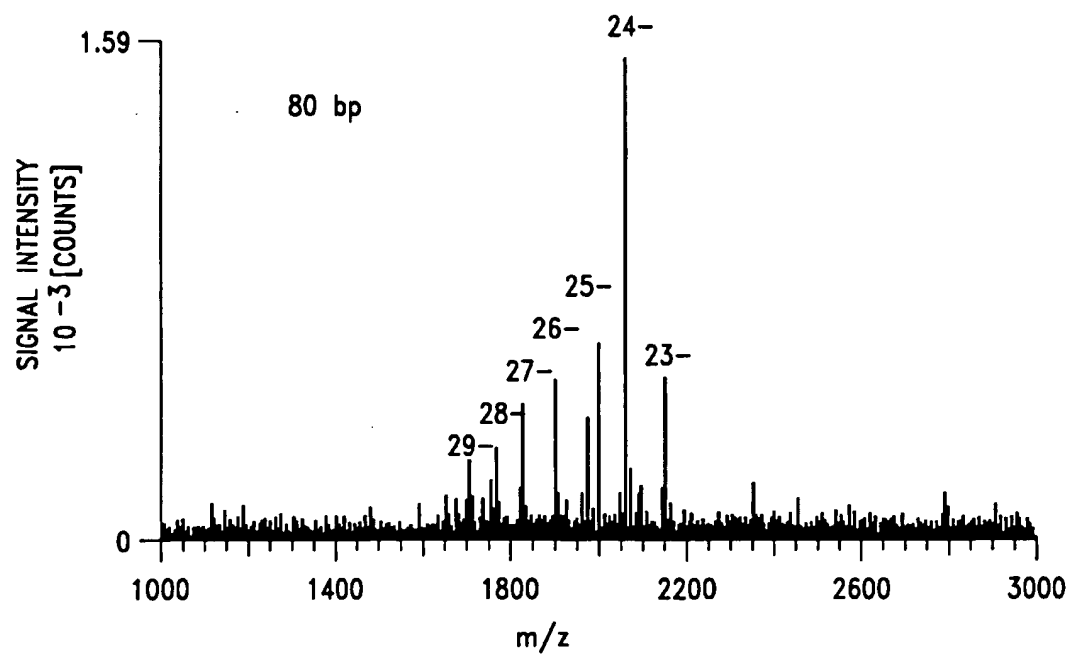
*FIG.-8*

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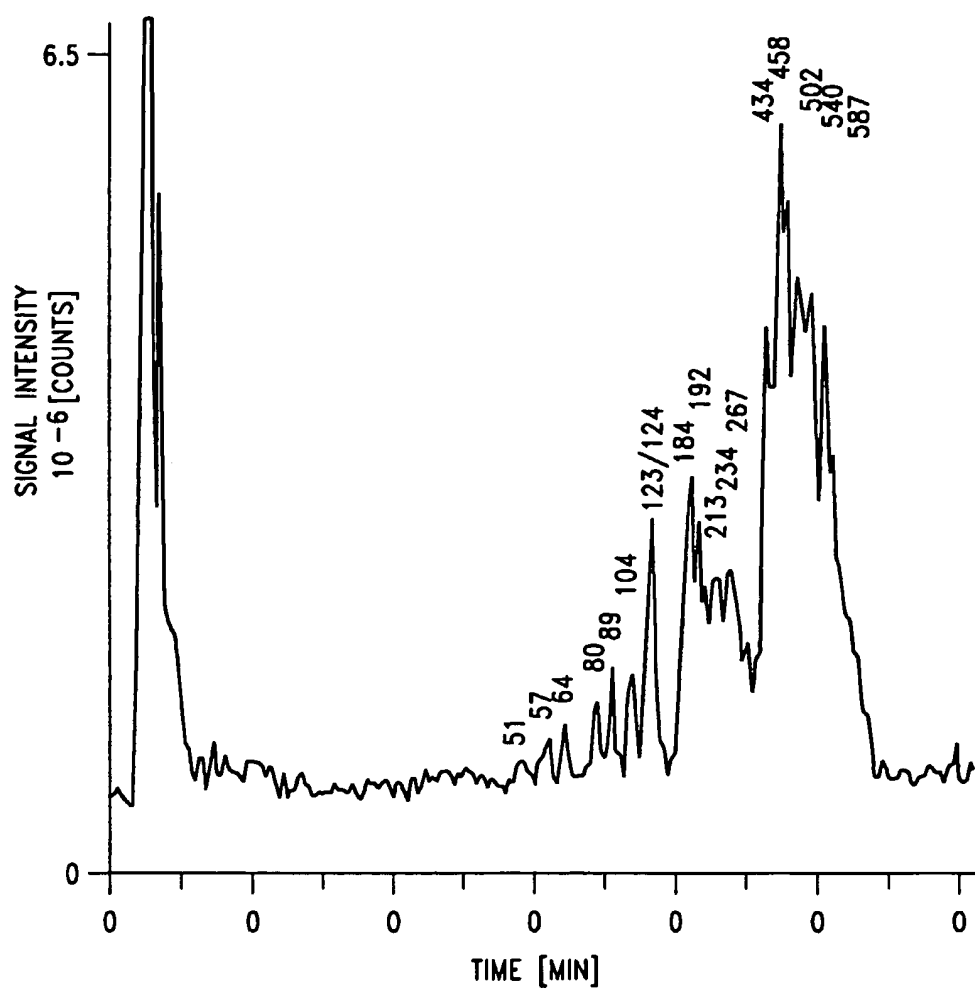
*FIG.-9**FIG.-10*

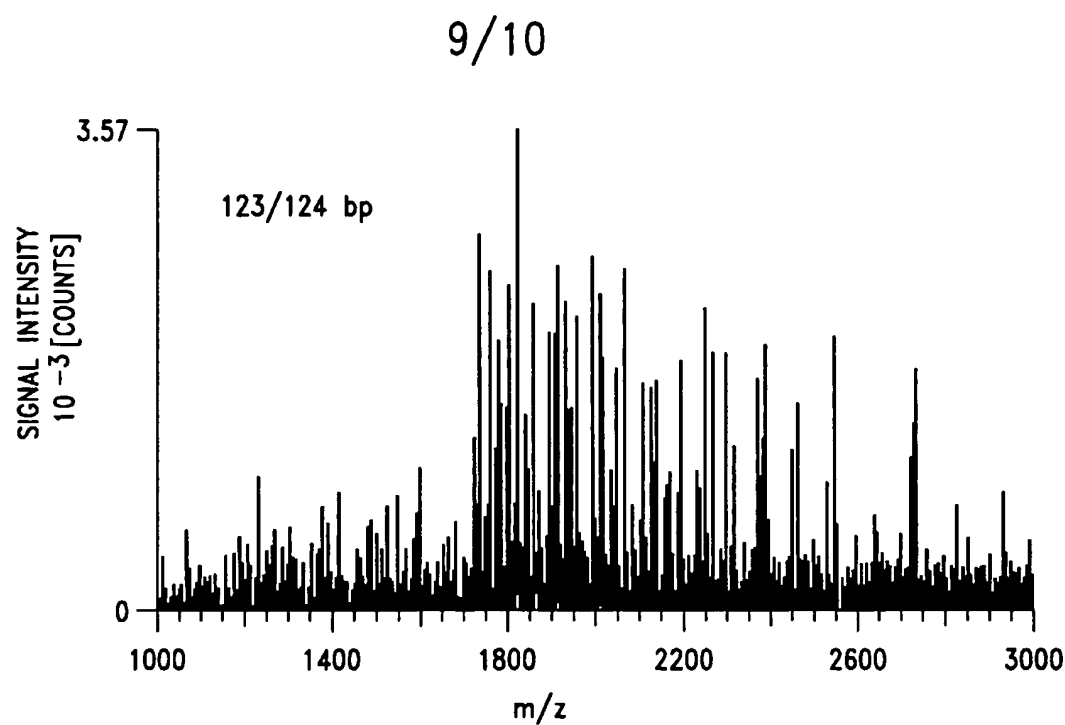
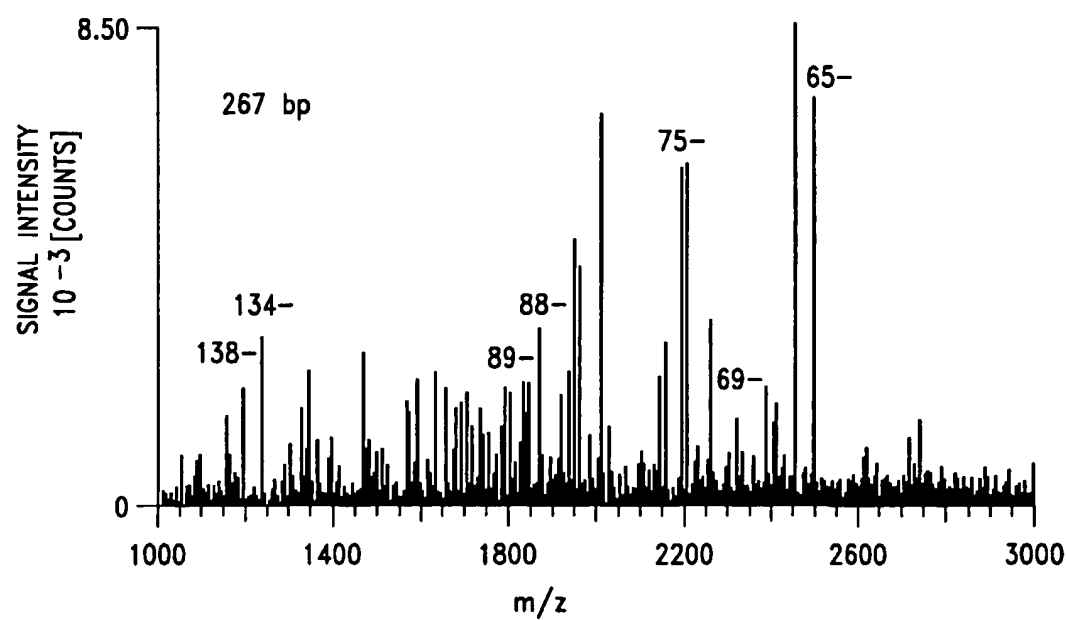
*FIG.-11**FIG.-12*

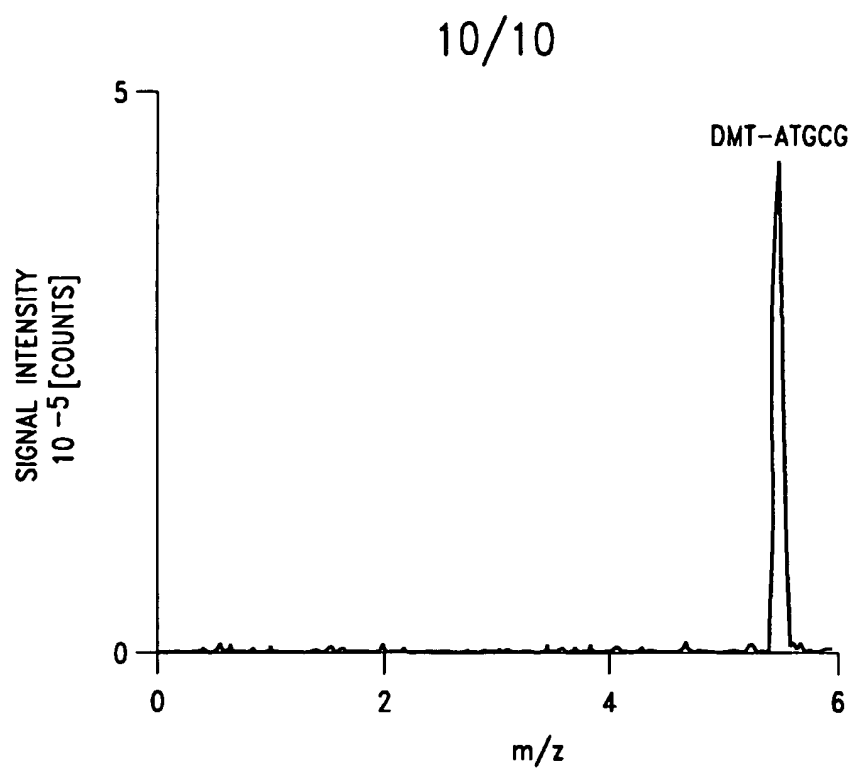
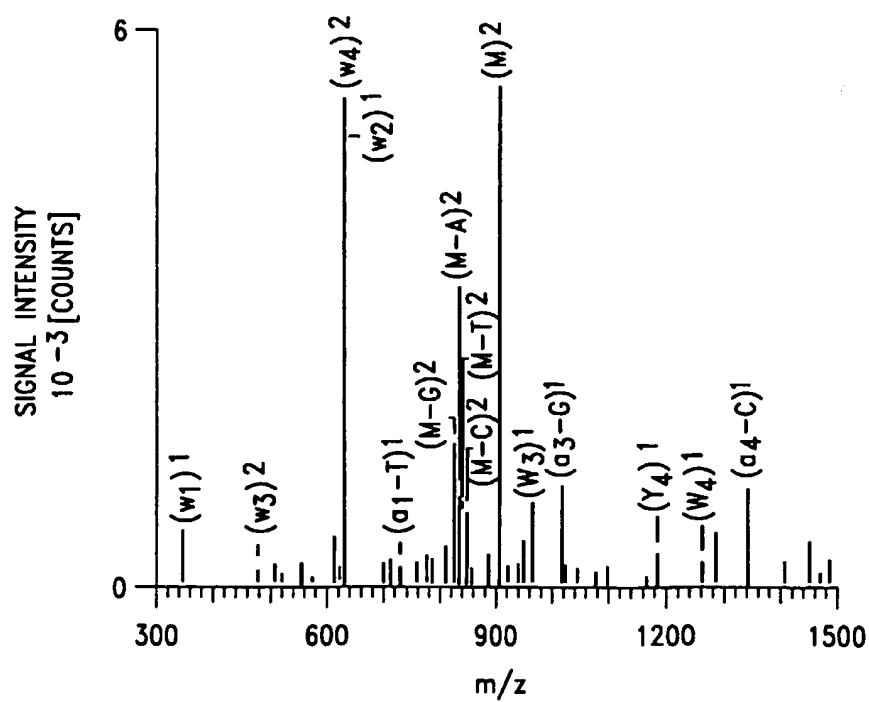
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*FIG.-13**FIG.-15*

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*FIG. - 14*

*FIG.-16**FIG.-17*

*FIG.-18**FIG.-19*

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/02539

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K1/36 B01D15/08 B01J20/26

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K G01N B01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                      | Relevant to claim No.                                                           |
|------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| A          | <p>US 5 972 222 A (TOGAMI DAVID W ET AL)<br/>26 October 1999 (1999-10-26)</p> <p>column 1, line 12-182<br/>column 2, line 24-45<br/>column 5, line 66 -column 6, line 13<br/>column 15, line 67</p> <p>---<br/>-/--</p> | <p>1,9,16,<br/>23,30,<br/>37,44,<br/>51,59,<br/>67,73,<br/>79,86,<br/>96,97</p> |



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents :

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Date of the actual completion of the international search

9 November 2001

Date of mailing of the international search report

20/11/2001

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/02539

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                 |
|------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| Category *                                           | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                                    | Relevant to claim No.                                                           |
| A                                                    | <p>HUBER C G ET AL: "Evaluation of volatile eluents and electrolytes for high-performance liquid chromatography-electrospray ionization mass spectrometry and capillary electrophoresis-electrospray ionization mass spectrometry of proteins - I. Liquid chromatography"</p> <p>JOURNAL OF CHROMATOGRAPHY A, ELSEVIER SCIENCE, NL, vol. 849, no. 1, 16 July 1999 (1999-07-16), pages 161-173, XP004173686</p> <p>ISSN: 0021-9673</p> <p>abstract</p> | <p>1,9,16,<br/>23,30,<br/>37,44,<br/>51,59,<br/>67,73,<br/>79,86,<br/>96,97</p> |
| A                                                    | <p>MOORE R E ET AL: "A MICROSCALE ELECTROSPRAY INTERFACE INCORPORATING A MONOLITHIC, POLY(STYRENE-DIVINYLBENZENE) SUPPORT FOR ON-LINE LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY ANALYSIS OF PEPTIDES AND PROTEINS"</p> <p>ANALYTICAL CHEMISTRY, AMERICAN CHEMICAL SOCIETY, COLUMBUS, US, vol. 70, no. 23, 1 December 1998 (1998-12-01), pages 4879-4884, XP002929947</p> <p>ISSN: 0003-2700</p> <p>abstract</p>                                  | <p>1,9,16,<br/>23,30,<br/>37,44,<br/>51,59,<br/>67,73,<br/>79,86,<br/>96,97</p> |
| A                                                    | <p>US 5 728 457 A (FRECHET JEAN ET AL)</p> <p>17 March 1998 (1998-03-17)</p> <p>column 2, line 35-60</p> <p>column 10, line 25</p>                                                                                                                                                                                                                                                                                                                    | <p>1,9,16,<br/>23,30,<br/>37,44,<br/>51,59,<br/>67,73,<br/>79,86,<br/>96,97</p> |
| A                                                    | <p>HUBER C G: "Micropellicular stationary phases for high-performance liquid chromatography of double-stranded DNA"</p> <p>JOURNAL OF CHROMATOGRAPHY A, ELSEVIER SCIENCE, NL, vol. 806, no. 1, 8 May 1998 (1998-05-08), pages 3-30, XP004121165</p> <p>ISSN: 0021-9673</p> <p>abstract</p>                                                                                                                                                            | <p>1,9,16,<br/>23,30,<br/>37,44,<br/>51,59,<br/>67,73,<br/>79,86,<br/>96,97</p> |
|                                                      | <p>---</p> <p>-/--</p>                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                 |

## INTERNATIONAL SEARCH REPORT

International Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                                                                                                               | Relevant to claim No.                                                           |
|------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| A          | <p>TENNIKOVA T B ET AL: "AN INTRODUCTION TO MONOLITHIC DISKS AS STATIONARY PHASES FOR HIGH PERFORMANCE BIOCHROMATOGRAPHY" JOURNAL OF HIGH RESOLUTION CHROMATOGRAPHY, WILEY VCH, WEINHEIM, DE, vol. 23, no. 1, January 2000 (2000-01), pages 27-38, XP000935789<br/>ISSN: 0935-6304<br/>abstract</p>                                                                                                                                                                                                                              | <p>1,9,16,<br/>23,30,<br/>37,44,<br/>51,59,<br/>67,73,<br/>79,86,<br/>96,97</p> |
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International Application No

PCT/US 01/02539

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